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Simultaneous gas chromatographic determination of 2,4-D and dicamba in human blood and urine

The compounds 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methoxy-3,6-dichlorobenzoic acid (dicamba) are herbicides commonly used to control a variety of broadleaf plants. An acute poisoning case was recently investigated by the Hawaii Community Study on Pesticides in which the victim intentionally ingested a formulation containing 2,4-D and dicamba. The occurrence of this attempted suicide created a need for a rapid and sensitive method for detecting these compounds in small amounts of human blood and urine.

Several methods for detecting 2,4-D residues in a variety of substrates were considered for the analysis of 2,4-D and dicamba in human blood and urine¹⁻³. While generally sensitive, these methods were neither rapid nor applicable to the relatively small samples available.

The Hawaii Community Study on Pesticides uses the method of BEVENUE *et al.*⁴ to determine pentachlorophenol (PCP) residues in human blood. In an evaluation done by this laboratory⁵, the procedure was shown to be rapid, accurate, sensitive and reproducible. Because 2,4-D and dicamba display chemical characteristics similar to those of PCP, these compounds are also detected by the procedure.

This note describes the application of the method of BEVENUE *et al.*⁴ to the simultaneous determination of 2,4-D and dicamba in human blood and urine.

Experimental

Solvents and reagents. The following solvents and reagents were used: benzene, hexane, isooctane (Mallinckrodt Nanograde Reagents), used as received; 0.1 N H₂SO₄, benzene extracted; 2,4-dichlorophenoxyacetic acid; 2-methoxy-3,6-dichlorobenzoic acid; N-methyl-N'-nitro-N-nitrosoguanidine, supplied by Aldrich Chemical Co., Milwaukee, Wisc.

The diazomethane solution⁶ was prepared as follows: hexane (25 ml) and 2 ml of 20% aqueous NaOH were combined in a 50-ml erlenmeyer flask and 0.5 g of N-methyl-N'-nitro-N-nitrosoguanidine was added in small increments. When the reaction was complete, the hexane layer was decanted into a small glass bottle equipped with a Teflon-lined screw cap. The diazomethane solution was kept in a freezer for up to one week. Because diazomethane is a skin irritant and a carcinogen, its preparation and use was confined to a well-ventilated hood.

Standard solutions. Stock solutions of 2,4-D and dicamba in benzene were prepared, each containing 1 mg of compound per ml of solvent. In separate 100-ml volumetric flasks 1.0 ml of each stock solution was treated with 0.5 ml of diazomethane solution and allowed to stand for 15 min. The solutions were then diluted to 100 ml with isooctane, thus giving solutions which contained 10 µg/ml of each compound as its methyl ester. From these solutions, working standards were prepared in appropriate concentrations.

Apparatus. Gas chromatographic determinations were made on a MicroTek MT 220 gas chromatograph equipped with a ³H electron capture detector and a 0.32 cm I.D. × 180 cm borosilicate glass column packed with a mixture of 4%

SE-30 and 6% QF-1 on Chromosorb W (AW-DMCS), high-performance grade, 80-100 mesh.

The instrument conditions were: inlet temperature 220°, detector temperature 208°, column temperature 190°; nitrogen flow rate 85 ml/min.

The 25-ml evaporative concentrator tube was Kontes No. 570050-2526, the evaporative concentrator column Kontes No. 569251-0324.

Extraction. One milliliter of blood serum or 5 ml of urine were combined with 20 ml of 0.1 N H₂SO₄ and 12 ml of benzene in a 125-ml glass-stoppered erlenmeyer flask equipped with a 1-in. Teflon-coated magnetic stirring bar and mixed for 20 min at 50° on a heated stirplate. After cooling, the contents of the flask were transferred to a 35-ml centrifuge tube and centrifuged for 15 min to effect phase separation. The benzene (top) layer was transferred to a 25-ml evaporative concentrator tube, and the aqueous phase was re-extracted three times with 5-ml portions of benzene. With each wash the mixture was shaken vigorously for 1 min, and the phases were separated by centrifugation. The benzene extracts were combined in the 25-ml evaporative concentrator tube, a boiling chip was added, an evaporative concentrator column was attached, and the combined extract was concentrated to 2 ml in a water-bath at 100°.

Methylation. Diazomethane solution (0.2 ml) was added to the concentrated extract, and the mixture was allowed to stand for 15 min. Excess diazomethane was then removed by gently bubbling dry nitrogen through the extract until the yellow color disappeared, and the volume was adjusted to 10.0 ml with isooctane.

Gas chromatography. The methylated extract was diluted as necessary, and 2 to 8 μ l were injected into the gas chromatograph. Following each extract injection, an amount of standard which gave a response of similar peak height ($\pm 10\%$) was injected.

Results and discussion

The 2,4-D and dicamba levels in the poisoning case samples are given in Table I. The results of a recovery study to determine the applicability of the procedure to the analysis of human blood and urine for 2,4-D and dicamba residues are given in Table II. The samples were fortified, prior to extraction, with 2,4-D and dicamba acids at levels which compared generally with the wide range of concentrations found in the poisoning case samples.

In all cases responses to 2,4-D and dicamba (as their methyl esters) were quantitated by comparison with standard responses in terms of peak height. By comparing responses with peak heights differing by 10% or less, quantitation problems associated with lack of linearity and changing sensitivity, sometimes encountered with electron capture detectors, were minimized.

The limits of detectability in 1 ml of blood serum were 0.03 p.p.m. for dicamba and 0.05 p.p.m. for 2,4-D. In 5 ml of urine the limits of detectability were 0.01 and 0.02 p.p.m. for dicamba and 2,4-D, respectively.

Under the conditions given, dicamba and 2,4-D methyl esters had retention times of 1.2 and 1.9 min, respectively; PCP methyl ether had a retention time of 2.2 min. Because the three compounds are well separated on the gas chromatographic column, the procedure is useful for detecting any or all of the compounds in the same sample.

TABLE I
2,4-D AND DICAMBA LEVELS IN POISONING CASE SAMPLES

Sampling	Blood serum		Urine		to Date*	Time	Vol. (ml)	2,4-D (p. p. m.)		Dicamba (p. p. m.)	
	Date*	Time	Date*	Time				Serum	Urine	Serum	Urine
1	10/14	7:00 a.m.	10/14	11:00 a.m.	10/14	3:00 p.m.	740	1006	145.2	46.4	215.0
2	10/14	3:00 p.m.	10/14	3:00 p.m.	10/14	11:00 p.m.	375	1031	274.0	22.3	280.3
3	No sample taken		10/14	11:00 p.m.	10/15	9:00 a.m.	1230	—	151.5	—	47.5
4	10/15	9:00 a.m.	10/15	9:00 a.m.	10/15	4:00 p.m.	—	911.9	762.0	8.52	12.8
5	10/15	4:00 p.m.	10/15	4:00 p.m.	10/15	11:00 p.m.	775	931.2	599.8	8.12	1.95
6	10/15	11:15 p.m.	10/15	11:00 p.m.	10/16	7:45 a.m.	1170	736.4	784.3	6.99	4.22
7	10/16	7:50 a.m.	10/16	7:45 a.m.	10/16	4:15 p.m.	720	648.6	408.9	4.47	1.08
8	10/16	4:15 p.m.	10/16	4:15 p.m.	10/16	11:00 p.m.	835	461.0	1333	3.66	0.95
9	10/16	11:00 p.m.	10/16	11:00 p.m.	10/17	9:00 a.m.	740	383.1	1900	2.54	1.35
10	10/17	9:00 a.m.	10/17	9:00 a.m.	10/17	5:00 p.m.	310	364.3	1442	1.97	1.06
11	10/17	5:00 p.m.	10/17	5:00 p.m.	10/18	10:00 a.m.	1640	72.4	409.6	1.30	0.80
12	10/18	10:00 a.m.	10/18	10:00 a.m.	10/18	4:30 p.m.	910	5.23	77.9	0.47	0.54
13	10/18	4:30 p.m.	10/18	4:30 p.m.	10/19	9:45 a.m.	1400	1.69	8.60	0.23	0.40
14	10/19	9:45 a.m.	10/19	9:45 a.m.	10/19	3:00 p.m.	450	1.78	3.88	0.15	0.25
15	No sample taken		10/19	3:00 p.m.	10/20	9:00 a.m.	2150	—	4.51	—	0.12
16	10/20	9:00 a.m.	10/20	9:00 a.m.	10/21	8:30 a.m.	2420	1.26	2.90	0.15	0.06
17	10/21	8:30 a.m.	10/21	8:30 a.m.	10/22	9:30 a.m.	3710	0.38	1.58	0.15	<0.01
18	10/22	9:30 a.m.	10/22	9:30 a.m.	10/23	8:00 a.m.	2600	0.16	1.74	0.03	<0.01
19	10/23	7:45 a.m.	10/23	8:00 a.m.	10/24	8:45 a.m.	4405	0.30	0.54	0.07	<0.01
20	10/24	8:45 a.m.	10/24	8:45 a.m.	10/25	1:00 p.m.	2660	0.88	0.71	0.06	0.03
21	10/25	1:00 p.m.	10/25	1:00 p.m.	10/27	10:45 a.m.	3800	0.38	0.25	<0.03	0.04
22	10/27	10:45 a.m.	10/27	10:45 a.m.	10/29	8:00 a.m.	2110	0.28	0.42	<0.03	<0.01
23	No sample taken		10/29	8:00 a.m.	10/31	1:00 p.m.	3030	—	1.15	—	<0.01
24	10/31	1:00 p.m.	10/31	1:30 p.m.	11/3	7:30 a.m.	1360	0.33	1.13	<0.03	<0.01
25	11/7	9:00 a.m.	11/7	6:00 a.m.	11/8	6:00 a.m.	1300	0.56	0.84	<0.03	<0.01

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TABLE II

RECOVERY OF 2,4-D AND DICAMBA ADDED TO HUMAN BLOOD SERUM AND URINE

Sample ^a	Fortification level (p. p. m)		Found (p. p. m.)		% recovery	
	Dicamba	2,4-D	Dicamba ^b	2,4-D ^b	Dicamba	2,4-D
Urine	—	—	< 0.01 ^c	< 0.02 ^c	—	—
	65.0	685	68.8	779	106	114
	13.0	137	12.5	132	96	96
	1.30	13.7	1.15	11.6	88	85
	0.13	1.37	0.11	1.35	85	98
	0.013	0.137	0.01	0.15	77	109
	0.006	0.068	< 0.01 ^c	0.07	—	103
Blood serum	—	—	< 0.03 ^c	< 0.05 ^c	—	—
	65.0	685	61.0	636	94	93
	6.50	68.5	5.91	68.9	91	101
	0.65	6.85	0.55	5.96	85	87
	0.065	0.685	0.07	0.60	108	88
	0.006	0.068	< 0.03 ^c	0.06	—	88

^a Each sample a single analysis.^b Detected as their methyl esters.^c Limit of detectability.

No interfering responses were encountered in any of the samples analyzed; however, the use of the diazomethane solution in amounts greater than those indicated in the procedure can lead to interferences due to impurities in the reagent.

Using this procedure, an experienced analyst can process approximately sixteen samples per day.

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Measurement of Endrin Following Epidemics of Poisoning

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308

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Between 3 June and 15 July 1967, four explosive outbreaks of acute poisoning with the insecticide endrin occurred in Doha, Qatar and Hofuf, Saudi Arabia. They resulted in the hospitalization of 874 persons and the death of 26 persons. The outbreaks probably caused a lesser degree of poisoning in many others who recovered without seeking medical aid. A thorough epidemiological study reported by Weeks (1) revealed that flour in a lower deck of each of two ships had been contaminated by an emulsifiable concentrate of endrin carried on an upper deck of the same hold. The episodes were unrelated, but leakage in each instance probably occurred when the insecticide was unloaded by net. The flour, which was taken off later at different ports, led to poisoning when separate lots of it were made into bread and eaten.

This paper reports only our chemical studies concerned with the outbreaks. It describes the method used, and summarizes the chemical evidence that endrin from contaminated flour was, in fact, the cause of illness. The paper also presents available measurements pertaining to the distribution of endrin and its rate of loss in man.

Methods

The samples were collected in each country by consultants sent by the World Health Organization. An organophosphate insecticide was thought by local authorities to be the cause of the illness. A WHO team found an insecticide formulation (BHC) on a shelf in a bakery in Doha, Qatar. This insecticide formulation was also suspected as the probable contaminant. Selected benzene¹ extracts of bread and flour were analyzed by flame photometry and electron-capture gas-liquid chromatography for organophosphates and for chlorinated hydrocarbons. The findings were negative for organophosphates and γ -BHC in the flour and bread. The insecticide formulation was found to contain 11.75% γ -BHC (1,2,3,4,5,6-hexachlorocyclohexane) as the active ingredient. However, a compound in the bread and flour was isolated and identified as endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-endo-5,8-dimethanonaphthalene). This identification was confirmed

¹Pesticide quality benzene used.

by electroconductometric gas-liquid chromatography, thin-layer and paper chromatography, infrared and NMR spectroscopy.

The experimental details are given in three parts: the extraction procedures, the gas chromatographic procedures, and the confirmational analysis.

Extraction. The bread samples were thoroughly ground in a mortar. These ground samples (150-875 mg) were quantitatively transferred to 15-ml glass-stoppered centrifuge tubes using 2 ml pesticide quality isopropanol and 4 ml pesticide quality benzene. They were extracted by shaking in a Scientific Products Delux Mixer.² After centrifugation, the isopropanol-benzene mixture was aspirated and transferred to another 15-ml centrifuge tube. Hexane-extracted distilled water was added. The benzene phase was separated from the aqueous isopropanol phase.

Flour samples were thoroughly mixed and extracted by the same procedure. The benzene extracts of the bread and flour samples were diluted or concentrated to appropriate volume and held for analysis by gas-liquid chromatography.

The plasma samples were extracted by the method of Dale *et al.* (2) and the urine samples by the method of Cueto and Biros (3).

A few samples of stomach wall, liver, and kidney taken at autopsy during the first outbreak in Doha, Qatar were the only tissues received. These samples were weighed (374-654 mg) and transferred to tissue grinders. Each sample was homogenized 3 times with 2 ml pesticide quality n-hexane. The hexane extracts were combined, centrifuged, concentrated to appropriate volumes, and held for analysis by gas-liquid chromatography.

Gas Chromatography. The gas chromatographic analyses were completed on a Micro Tek Model MT 220 instrument and a Varian Model 1525 equipped with tritium electron-capture detectors. The Micro Tek MT 220 instrument was operated with a 4' x 1/4" aluminum column packed with 3% Dow 220 on 70/80 mesh, acid-washed, DMCS-treated, Chromosorb G. The temperatures of the column, the inlet injection port, and the detector were 175°C, 200°C, and 200°C, respectively. The carrier gas was nitrogen at 60 psi with a flow rate of 60 cc/min. The detector was operated in the d.c. mode (15 v). The Varian instrument was operated with a 5' x 1/8" Al column packed with 3% OV-17 on 70/80 mesh, acid-washed, DMCS-treated, Chromosorb G. The temperatures of the column, the inlet injection port, and the detector were 175°C, 198°C, and 200°C, respectively. The carrier gas was nitrogen at 60 psi with a flow rate of 120 cc/min. The detector was operated at a fixed potential (d.c. 90 v). Other

²Use of trade names is for identification purposes only, and does not constitute endorsement by the Public Health Service, or the U.S. Department of Health, Education, and Welfare.

columns used were 3% QF-1 and 3% DEGS. Best results were achieved on the 3% OV-17 and 3% Dow 200 columns. The endrin standard gave three peaks on the 3% Dow 200 column with retention times of 9.0, 17.4, and 20.2 minutes, respectively. This same pattern was observed in the sample extracts. Possible chemical degradation of the compound on the column might account for the presence of three peaks. On 3% OV-17 one peak was observed at 14.5 minutes.

Confirmational Analysis. The identification of the contaminant (endrin) was confirmed by subjecting flour extracts to electroconductometric gas-liquid, thin-layer, and paper chromatography. A Micro Tek Model 2000 instrument equipped with a Coulson Electrolytic Conductivity Detector operated in the oxidative mode for chloride determination was used. The column and other operating parameters were the same as those with the Micro Tek MT-220 instrument. The observed retention times were identical to those mentioned earlier.

Five grams of flour were placed in a 15-ml centrifuge tube, 8 ml isopropanol or 6 ml acetone was added, and the mixtures were shaken vigorously. The tubes were then centrifuged at 2000 rpm for 5 minutes. Paper chromatography of spots from 100 μ l aliquots of the supernatant liquid detected 50 ppm of endrin. Concentration of a 2.0-ml aliquot to 0.2 ml before spotting 100 μ l increased the sensitivity to 5 ppm. The paper chromatograms were developed by two-phase ascending systems including:

System A, Immoblie: 5% v/v paraffin oil in ether. Mobile: Acetone - water (3:1). Development time, 1 hour.

System B, Immoblie: N,N'-dimethylformamide, 35% v/v in ether. Mobile: 2,2,4-Trimethylpentane. Development time, 45 minutes.

System C, Immoblie: 5% v/v paraffin oil in ether. Mobile: Methyl alcohol-acetic acid-water (80:5:15). Development time, 1 hour.

The spots on the chromatograms were detected by Mitchell's spray for chloride compounds (0.4 g silver nitrate, 2.5 ml 2-phenoxy-ethanol, and 5 ml acetone, diluted to 50 ml with chloride-free water) (4). The R_f values corresponded to those for standard endrin and were 0.53, 0.89, and 0.45 for systems A, B, and C, respectively.

Thin-layer chromatography was accomplished on aluminum oxide and silica gel plates using pesticide quality n-hexane-ethyl ether (95:5) for development. The R_f values for both samples and standard endrin were 0.35 on aluminum oxide and 0.31 on silica gel.

Verification that the flour sample extracts contained endrin was obtained by spotting the extracts on paper along with separate spots of standard solutions of endrin, dieldrin and a mixture of the two. These papers were then exposed to ultraviolet light for 1 hour. Two-phase chromatography of the papers by two systems of

TABLE 1

Endrin Concentrations Found in Various Samples
From Three of the Four Outbreaks

Sample	Endrin concentration (ppm)		
	First Doha outbreak	Third Doha outbreak	Hofuf outbreak
Bread, portions of which had been eaten by patients	48	1,339-1,807	243-537
Flour, used to make the same bread	INA ^a	2,153	3,367
Blood from patients	0.007-0.032	INA	0.008-0.027
Urine from patients	INA	INA	<0.004-0.007
Vomitus from patient	INA	INA	5.24
Tissues (autopsy) from:			
stomach wall	0.16	INA	INA
liver	0.685	INA	INA
kidney	0.116	INA	INA

^a INA: Information not available.

TABLE 2

Concentration of Endrin in the Urine and Plasma
Collected from Acutely Poisoned Patients on the Day of Onset

Patient's initials	Interval from ingestion to symptoms (hr)	Concentration of endrin (ppm)	
		Plasma	Urine
H.N.	3.3	0.008	0.005
F.D.	1.5	0.011	<0.007
A.R.	1.5	0.027	<0.004

different polarity (4) verified that the flour extracts gave the same decomposition products upon exposure to U.V. light as were given by standard endrin.

Our results were confirmed independently at the pesticide laboratory at Perrine, Florida by three separate methods. Portions of flour, bread, and other samples were subjected to thin-layer chromatography, infrared spectrophotometry, and nuclear magnetic resonance spectrometry. The thin-layer chromatograms, IR spectra, and NMR spectra of the samples were compared with the thin-layer chromatograms, IR spectra and NMR spectra of pure endrin and of pure endrin that had been subjected to weathering. Virtually all spots on sample chromatograms were accounted for on the chromatograms of weathered endrin. All bands on the IR spectra of the samples were accounted for on the IR spectra of the weathered endrin also, as were the peaks on the NMR spectra. There was no doubt that the chromatograms and the spectra of the samples were those of endrin or its degradation products.

Results and Discussion

The flour used for making bread was not necessarily the most contaminated. The concentration of endrin in cloth from a stained area of one sack was 194,000 ppm; the concentration in flour from directly beneath the stain was 28,070 ppm; and the concentration in flour from deep within the sack was 4,460 ppm. As shown in Table I, the highest concentration of endrin found in flour known to have been used for making bread eaten by a patient was 3,367 ppm. The lowest concentration of endrin in bread known to have caused poisoning in one of the four outbreaks was 48 ppm. The lowest comparable value of 176 ppm was reported earlier by Coble *et al.* (5). Table I shows that endrin was measured not only in flour, bread, and vomitus, but also in blood, urine, and tissues from patients. Endrin is not found in comparable samples from the general population or even from workers who manufacture and formulate the insecticide (6).

We received samples of urine and plasma which had been obtained from three acutely poisoned patients on the day of onset of symptoms. Table II shows that endrin was more regularly detected in the plasma and that the concentration was higher than in the urine. The 14 late samples that we received were uniformly negative for endrin. By contrast, dieldrin is easily measured in the blood of people in the general population (2) and was still elevated 14 months after accidental ingestion leading to poisoning (7).

Dieldrin, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, and BHC were detected in the blood, urine, and tissue (stomach wall). The levels were no greater than those commonly found in the general population, and these compounds were entirely unrelated to the illness.

The chemical evidence presented clearly indicates that endrin was in fact the contaminant that caused the illness and deaths in the four outbreaks of poisoning in Doha, Qatar and Hofuf, Saudi Arabia. Endrin was detected in the blood and urine of patients, and in tissues taken at autopsy. The concentrations in the blood were similar to those found by Coble *et al.* (5). Samples of blood and urine taken 29-31 days after the outbreak were uniformly negative for endrin.

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Dr. Wayland J. Hayes, Jr., formerly with the PHS Pesticides Program, gave advice and guidance throughout the study. Dr. Hayes is now with the Department of Biochemistry, Vanderbilt University, Nashville, Tennessee.

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A Method for Determination of Low Levels of Exposure to 2,4-D and 2,4,5-T

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478

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A method has been developed for the determination of trace quantities of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenol (2,4-DCP), and 2,4,5-trichlorophenol (2,4,5-TCP) in human and rat urine. The method involves acid hydrolysis of the phenolic conjugates, extraction of the free phenols and acids, ethylation with diazoethane, silica-gel column chromatography clean-up of the derivatized urine extract, and gas chromatographic determination using the electron-capture detector. The average recoveries of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP from rat urine spiked with known amounts of the herbicides and their phenols were 94%, 98%, 92%, and 90%, respectively. The limits of detection for 2,4-D, 2,4,5-T, DCP, and TCP in rat urine were: 0.05, 0.01, 0.10, and 0.01 ppm, respectively. The method was used to analyze urine of rats given various levels of 2,4-D and 2,4,5-T by gavage. Results showed that levels of exposure of 3.75 mcg/kg for 2,4-D and 5.0 mcg/kg for 2,4,5-T in rats can be detected in urine within 24 hr from exposure. Urine samples from occupationally exposed people were analyzed and found to contain 0.2 to 1.0 ppm 2,4-D and 0.05 to 3.6 ppm 2,4,5-T.

INTRODUCTION

A number of derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are applied extensively as selective herbicides in the control of terrestrial and aquatic broadleaf plants. Because

of the widespread use of these compounds and their relatively lengthy persistence, particularly in treated lakes and streams,¹ potential human exposure may occur via several routes. These include consumption of contaminated plants, livestock, and water, as well as the direct exposure of agricultural spraymen and herbicide formulators. For toxicological and environmental monitoring of these herbicidal compounds, a rapid, sensitive procedure is needed for the detection in human and animal urine of the free acids and the chlorinated phenol degradation products which result from exposure.

Several methods are described in the literature for the gas chromatographic determination of the methyl ester derivatives of 2,4-D and 2,4,5-T isolated as residues from a variety of natural materials.²⁻⁶ Body tissues of sheep have been analyzed for 2,4-D by electron-capture gas chromatography of the free 2,4-dichlorophenol (2,4-DCP) formed by enzymatic hydrolysis of the herbicide residue.⁷ In addition, 2,4,5-T residues derivatized as the methyl ester have been determined in sheep urine⁶ by microcoulometric gas chromatographic analysis preceded by a Florisil column clean-up procedure. However, the data reported in both studies were based entirely on the analysis of fortified samples.

The purpose of this investigation was to develop a procedure for the simultaneous determination of trace quantities of 2,4-DCP, 2,4,5-trichlorophenol (2,4,5-TCP), 2,4-D, and 2,4,5-T in animal urine. The level of the free acids of 2,4-D and 2,4,5-T excreted in the urine of exposed animals can be used as an index of exposure to these herbicides.^{8,9}

EXPERIMENTAL

Apparatus and equipment Micro-Tek gas chromatograph, model MT 220, with a tritium electron-capture detector operated under the following conditions: a glass column, 180 cm $\times$ 0.4 cm i.d., packed with 20% OV-101 on Gas-Chrom Q, 60/80 mesh; nitrogen carrier gas with a flow rate of 60 ml/min; column temperature 175°C; injection port 210°C; detector 210°C; transfer line 250°C. Another glass column, 180 cm $\times$ 0.4 cm i.d. packed with 4% SE-30 and 6% QF-1 or 4% SE-30 and 6% OV-210 on Chromosorb W, high performance, 80/100 mesh can be used at the same operating temperatures and flow rates mentioned above, where 2,4-D and 2,4,5-T will have shorter retention times, 7 and 12 minutes, respectively. The 20% OV-101 column can also be operated at 195°C, with a flow rate of 60 ml/min., to produce retention times of approximately 5 minutes for 2,4-D and 9 minutes for 2,4,5-T.

Chromatographic Columns Size 22, i.d. 7 mm, length 200 mm, Kontes No. 420100.

Standards Analytical standards of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP were obtained from the Dow Chemical Co., Midland, Mich. The standards were used as received after the ethyl esters were checked for purity by gas chromatography.

Reagents Benzene and hexane, Mallinckrodt Nanograde solvent or equivalent. *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine, Aldrich Chemical Co., Milwaukee, Wis.

Silica gel, Woelm, activity grade I, Waters Associates, Inc., Framingham, Mass. Dry adsorbent for 48 hr at 170°C and store in a desiccator. On the day of use, deactivate 1 g of the silica gel by adding 1.5% by weight of water in the following manner. Add 15 ml water and 1 g silica gel to a 125-ml Erlenmeyer flask. Stopper and rotate the flask until the water is evenly distributed throughout the adsorbent. Allow to equilibrate for 2 to 3 hr with periodic shaking. Prepare the chromatographic columns just before use.

Deionized or distilled water, extracted with benzene, is used throughout the procedure.

Preparation of ethylating reagent

Prepare diazoethane reagent ⁴ as follows: Dissolve 2.3 g potassium hydroxide in 2.3 ml water in a 125-ml Erlenmeyer flask and allow the solution to cool to room temperature. Add 25 ml hexane and cool the flask in a freezer for 15 min. In a well-ventilated hood, add 1.6 g *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine in small portions and mix the contents of the flask after each addition. Decant the hexane layer into a bottle with a Teflon-lined screw cap. The diazoalkane solution may be stored at -20°C for as long as one week. Because of their demonstrated carcinogenicity, do not allow the nitrosoguanidine or the diazoethane solution to come in contact with the skin. Avoid the use of ground glass-stoppered containers or etched glassware for storing or preparing the reagent.

Preparation of standard solutions

Prepare stock standard solutions of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T in benzene by weighing 100 mg of each into separate 100-ml volumetric flasks. Dilute to volume with benzene. From these stock standards prepare an ethylated stock standard mixture by pipetting 2 ml 2,4-DCP, 0.1 ml 2,4,5-TCP, 0.5 ml 2,4-D, and 0.2 ml 2,4,5-T into a 50-ml volumetric flask. Add 15 ml benzene and then add diazoethane reagent, dropwise with a disposable pipet, until a definite yellow color persists. (Diazoethane reagent must be used in a well-ventilated hood.) Let stand 15 min; then bubble nitrogen through the solution until the yellow color disappears (5-10 min). Dilute to

volume with benzene. The ethylated stock standard mixture will contain, per milliliter, 40 mcg 2,4-DCP, 2 mcg 2,4,5-TCP, 10 mcg 2,4-D, and 4 mcg 2,4,5-T.

Preparation of standard curves

Pipet aliquots ranging from 0.05 to 1 ml from the ethylated stock standard mixture into separate 5-ml volumetric flasks. Dilute to volume with benzene. Five-microliter injections from these dilutions will contain 2.0–40.0 ng 2,4-DCP, 0.1–2.0 ng 2,4,5-TCP, 0.5–10.0 ng 2,4-D, and 0.2–4.0 ng 2,4,5-T. Separate calibration curves must be prepared for each compound. With an electrometer setting of 10×32 the following amounts will give approximately a 50% full-scale deflection on the recorder: 14 ng 2,4-DCP, 0.8 ng 2,4,5-TCP, 5 ng 2,4-D, and 1.7 ng 2,4,5-T. The sensitivity of the detector to the ethylated derivatives is 0.20 ng 2,4-DCP, 0.02 ng 2,4,5-TCP, 0.10 ng 2,4-D, and 0.04 ng 2,4,5-T.

Elution of ethylated 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T from silica gel

Determine the elution pattern of the four ethylated compounds before using the silica-gel column chromatography for clean-up of ethylated urine extracts. To a glass chromatographic column lightly plugged with glass wool and containing 1 g of the partially deactivated silica gel (1.5% water), add 10 ml hexane to prewash the column. When the last of the hexane has reached a point approximately 2 cm above the silica gel, add 0.3 ml of the ethylated standard stock mixture and begin collecting the eluate. Elute successively with 10 ml of each of the solvents listed in Table I, collecting each fraction separately. Inject 5 or 10 mcl from each fraction into the gas chromatograph and calculate the per cent of each compound present in the fraction. A typical elution pattern is shown in Table I.

TABLE I
Elution pattern of ethylated compounds from silica-gel column

Eluting solvents	2,4-DCP 20 mcg	2,4,5-TCP 1 mcg	2,4-D 5 mcg	2,4,5-T 2 mcg
20% Benzene-hexane	100%	100%	0	0
40% Benzene-hexane	0	0	0	0
60% Benzene-hexane	0	0	0–2%	20–25%
80% Benzene-hexane	0	0	98–100%	75–80%
100% Benzene	0	0	0	0

Analysis of urine

Pipet 1 to 5 ml (the actual volume may be determined by the anticipated residue level) of urine into a 25-ml concentrator tube. Add dropwise a volume of concentrated HCl equal to one-fifth the amount of urine, and mix well. Fit a stoppered reflux condenser to the tube and heat in a steam bath for 1 hr while cooling the condenser with circulating ice-water. Remove from the bath, cool, and rinse the sides and tip of the condenser with a total of 3 ml benzene. Mix contents of tube vigorously on a Vortex mixer for 1 min, then centrifuge, and transfer the benzene layer to a 15-ml centrifuge tube with a disposable pipet. Repeat the extraction with an additional 3-ml volume of benzene, adding the second benzene extract to the centrifuge tube. Add diazoethane dropwise with a disposable pipet until the yellow color persists. Let the solution stand 15 min; then bubble clean, dry nitrogen through the solution to remove the excess reagent. Concentrate the ethylated urine extract to 0.3 ml (± 0.1 ml) under a gentle stream of nitrogen at 40°C in a water bath.

Prepare a silica-gel chromatographic column as previously described. Prewash the column with 10 ml hexane and discard the washing. When the hexane level reaches a point approximately 2 cm above the silica gel, transfer

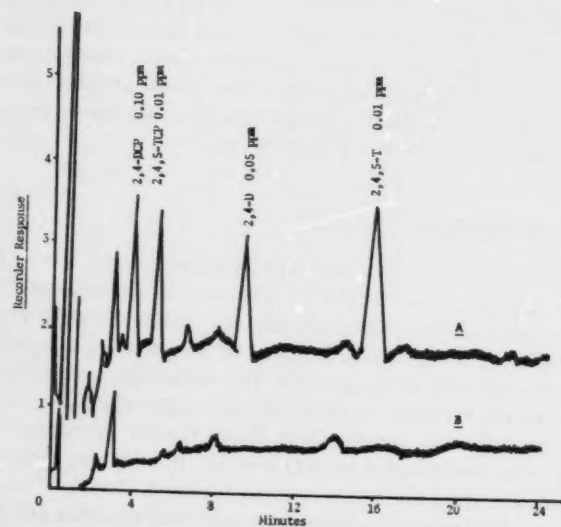


FIGURE 1 Representative chromatograms at a column temperature of 175°C. A, fortified control rat urine; B, control rat urine.

the concentrated urine extract to the column and begin elution with 10 ml of 20% benzene in hexane. This fraction contains ethylated 2,4-DCP and 2,4,5-TCP. Follow with 10 ml of 40% benzene-hexane and discard this fraction. Finally, add 10 ml of 60% benzene-hexane followed by 10 ml of 80% benzene-hexane and collect these fractions in a single tube. The combined 60% and 80% fraction contain ethylated 2,4-D and 2,4,5-T. Adjust the volume of each fraction to 10 ml with benzene. If the anticipated residue level is low, the fractions may be concentrated to 5 ml under a gentle stream of nitrogen at 40°C in a water bath. Inject into the gas chromatograph 5–10 mcl of the 20% fraction for the determination of the phenols and 5–10 mcl of the combined 60–80% fraction for the determination of the chlorophenoxyacetic acids. The elution pattern of the four compounds extracted from a fortified urine sample must be established and should be the same as previously indicated. The limits of detection for 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T are 0.10, 0.01, 0.05, and 0.01 ppm, respectively. A typical chromatogram is shown in Figure 1.

RESULTS AND DISCUSSION

The ethylated derivatives of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T were selected because of their stability and minimal hydrolysis during sample manipulation and silica-gel column chromatography. The gas chromatographic resolution of the phenols having short retention times from those peaks which interfere with them was improved by ethylation as compared to the more commonly used methyl derivatization.

Hydrolysis conditions

Urine samples from rats which had been fed 2,4-D and 2,4,5-T (10^{-1} the LD_{50}) were analyzed to determine the optimum hydrolysis conditions for complete recovery of herbicide residues. The samples were acidified and extracted without heat hydrolysis, or were hydrolyzed in a steam bath for 10, 60, 90-min intervals and then extracted. The derivatives were prepared and cleaned up, and the herbicide residues were quantitatively determined by gas chromatography. Results are shown in Table II. The recoveries increased significantly when the samples were heated for 10 min, and then remained relatively constant up to the 90-min hydrolysis period. One hour was selected as the optimum time of heat hydrolysis because the extraction efficiency was maximum at this time. In addition, fewer emulsification problems during the extraction step were observed for samples hydrolyzed for 1 hr.

TABLE II

The effect of acid hydrolysis time on recovery of herbicide residues from rat urine^a

Compound	Room temperature	100°C			
		10 min	30 min	60 min	90 min
2,4-D	10300	12500	12400	12700	12600
2,4,5-TCP	356	448	434	460	402
2,4,5-T	6350	7745	7550	8910	8010

^aRats were fed 10^{-1} the LD₅₀. Results are expressed in micrograms.

Recovery studies

Recovery studies were performed using fortified control rat urine. Five-milliliter samples of rat urine were spiked with different levels of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T in aqueous NaOH. The samples were hydrolyzed with acid for 1 hr at 100°C, extracted, derivatized, cleaned up by silica-gel chromatography, and analyzed gas-chromatographically by the described procedure. The levels of fortification and recovery data for each compound are shown in Table III.

TABLE III

Recovery of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T from 5 ml of fortified rat urine^a

2,4-DCP		2,4,5-TCP		2,4-D		2,4,5-T	
Added mcg	Recovered %	Added mcg	Recovered %	Added mcg	Recovered %	Added mcg	Recovered %
20.0	89-91	1.0	91-95	5.0	84-88	2.0	104-107
10.0	94-96	0.5	85-90	2.5	96-98	1.0	97-99
4.0	87-89	0.2	85-86	1.0	92-96	0.4	93-97
2.0	94-96	0.1	91-94	0.5	97-99	0.2	94-96

^aAverage of three determinations.

Application of the method for the determination of low-level exposure to 2,4-D and 2,4,5-T

Male Sprague-Dawley rats weighing 420 to 470 g were dosed by gavage with 10^{-1} , 10^{-2} , 10^{-4} , and 10^{-5} of the LD₅₀ of 2,4-D and 2,4,5-T. The LD₅₀ of 2,4-D was considered to be 375 mg/kg and of 2,4,5-T, 500 mg/kg.¹⁰ The doses were administered daily for 3 days to two rats at each dose level. The

animals were maintained in stainless steel metabolism cages (Acme Metal Products, Inc., Chicago, Ill.); the two rats given the same dose level were maintained in the same cage. The urine samples were collected in a manner which minimized fecal contamination before the exposure period and at timed intervals during and after exposure. The described procedure was used to determine 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T in the urine, and the results were calculated in terms of micrograms excreted in 24 hr. The excretion rates of 2,4-D and 2,4,5-T are shown graphically in Figures 2 and 3. The total dosages and urinary excretion of the different levels of exposure for both 2,4-D and 2,4,5-T are shown in Table IV.

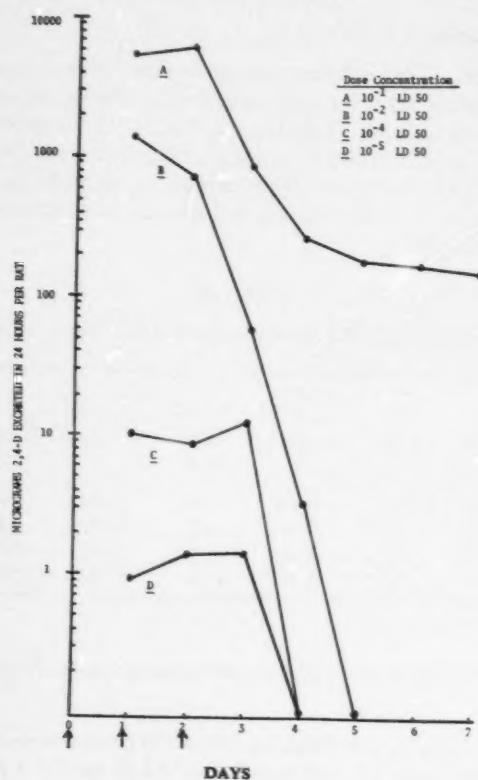


FIGURE 2 The rate of urinary excretion of 2,4-D from rats fed three consecutive doses. Arrows indicate time of dosage.

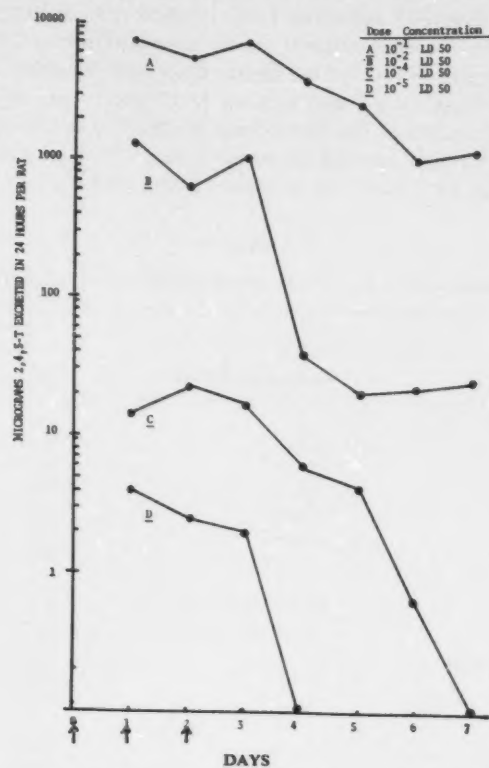


FIGURE 3 The rate of urinary excretion of 2,4,5-T from rats fed three consecutive doses. Arrows indicate time of dosage.

TABLE IV

The relation between total dosage and urinary excretion of 2,4-D and 2,4,5-T

Total dosage mg/rat		Total mg in urine/rat		No. of days for total excretion		Percent of total dose excreted in urine	
2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T
48.6	64.5	13.82	31.494	14	35	28	49
4.36	6.45	2.24	3.2135	4	12	46	50
0.0507	0.0645	0.0319	0.0570	3	6	63	88
0.00507	0.00645	0.0038	0.0065	3	3	75	100

The data in Figures 2 and 3 and Table IV show that the excretion rates of 2,4-D and 2,4,5-T were dependent on the dose administered. The per cent excretion decreased in rats fed the higher doses; this finding is in agreement with those of Clark *et al.*⁸ and Khanna and Fang,⁹ who reported similar results in their studies of the metabolism of 2,4-D. The excretion of 2,4-D and 2,4,5-T in urine of rats fed the lowest level (3.75 mcg/kg/day for 2,4-D; 5.00 mcg/kg/day for 2,4,5-T) was complete 2 days after the final dosage.

TABLE V

Results of analysis of urine from people occupationally exposed to 2,4-D and 2,4,5-T^a

Sample	Exposure compound	Results (ppm)		
		2,4-D	2,4,5-T	
Spray operator				
1	2,4,5-T	N.D. ^b	1.1	
2	2,4,5-T	N.D.	2.8	
3	2,4,5-T	N.D.	2.6	
4	2,4,5-T	N.D.	3.6	
5	2,4,5-T	N.D.	3.0	
6	2,4,5-T	N.D.	1.3	
Farmer				
7	2,4-D and 2,4,5-T	0.20	N.D.	
8	2,4-D and 2,4,5-T	0.19	N.D.	
Foreman, spray crew				
9	2,4,5-T	N.D.	1.2	
10	2,4,5-T	N.D.	0.5	
Herdsman				
11	2,4-D	N.D.	N.D.	
12	2,4-D	N.D.	N.D.	
Farm laborer				
13	2,4-D	N.D.	N.D.	
14	2,4-D	N.D.	N.D.	
15	2,4-D	N.D.	N.D.	
16	2,4-D	N.D.	N.D.	
Pesticide project officer				
17	2,4-D	N.D.	N.D.	
18	2,4-D	N.D.	N.D.	
Spray operator				
19	2,4-D	1.0	N.D.	
20	2,4-D	0.2	N.D.	
Aircraft spray operator				
21	2,4-D and 2,4,5-T	0.4	0.05	
22	2,4-D and 2,4,5-T	1.0	0.05	

^aThe derivative is unknown.

^bN.D., not detected.

In all rats given 2,4-D, the major portion of the administered herbicide was excreted as the unchanged compound in the urine. Under the conditions of the described procedure it was not possible to detect the presence of metabolized residues. On the other hand, rats given 50 mg/kg of 2,4,5-T excreted at least three metabolites in addition to 2,4,5-T. 2,4,5-TCP was detected at high levels of exposure and represented about 0.5 to 9.0% of the 2,4,5-T excreted at a given time following initial dosage. The level of 2,4,5-TCP in the urine decreased more rapidly with time as compared with 2,4,5-T, and no detectable amounts of 2,4,5-TCP were found for the low levels of exposure (10^{-4} and 10^{-5} the LD_{50}), indicating that the determination of 2,4,5-TCP cannot be used as an index of exposure to low levels of 2,4,5-T. Mass spectrometric analysis of the two additional metabolites indicated that one is a hydroxylated trichlorophenoxyacetic acid and the other is a hydroxylated trichlorophenol. Further confirmation by comparison with authentic materials is required. The presence of 2,4-D, 2,4,5-T, and 2,4,5-TCP in rat urine was confirmed by gas chromatography, silica-gel column chromatography employing selective differential elution, and combined gas chromatography-mass spectrometry of the derivatized residues isolated from rat urine and compared with authentic compounds.

To demonstrate the applicability of the method for monitoring occupational exposure, urine from people directly and indirectly involved in the application of 2,4-D and 2,4,5-T derivatives was analyzed. The results, as shown in Table V, indicate a higher degree of exposure for spray operators than those whose occupations afforded less direct contact.

It can be generally concluded that the method suggested for the determination of 2,4-D and 2,4,5-T in urine may be used to determine low levels of exposure to these herbicides. Levels of exposure of 3.75 mcg/kg for 2,4-D and 5.00 mcg/kg for 2,4,5-T in rats can be determined in urine within 24 hr from exposure.

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Relative Retention Ratios of Ninety-Five Pesticides and Metabolites on Nine Gas-Liquid Chromatographic Columns Over a Temperature Range of 170 to 204°C in Two Detection Modes

PESTICIDE
PUBLICATION

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Tables of relative retention data are presented for 95 organochlorine and organophosphorus pesticidal compounds at column oven temperatures from 170 to 204°C in 2° increments. Retention values, relative to aldrin or parathion ($RRT_{A \text{ or } P}$), were found to vary inversely with increasing column temperature for all compounds eluting later than the reference compound, and in direct proportion for those compounds eluting earlier than the fixed reference compound. Studies revealed a linear relationship between column temperature and relative retention ratios; thus, by obtaining precise values by gas-liquid chromatography for a number of pesticidal compounds at 170, 185, and 200°C, a computer program was developed to obtain $RRT_{A \text{ or } P}$ values at all 2° increments from 170 to 204°C, the customary limits in the gas-liquid chromatography of pesticides. The tables are valuable in the tentative identification of pesticidal peaks resulting from the chromatography of sample extracts for multiresidue assessment.

A number of authors have published relative retention data of pesticidal compounds obtained on various gas-liquid chromatographic (GLC) columns by different modes of detection (1-7). In each of the cited publications, the relative retention time (RRT) data have been obtained at one given temperature for each column used.

These data have proved valuable, provided that the researcher carefully adjusts the column temperature to the same point as that reported by the author. In some instances, this is difficult, since the readout pyrometer on many gas chromatographs may be grossly in error. When this occurs unbeknown to the chromatographer, the resulting RRT values, particularly those for

late eluting compounds, will be significantly different from those reported in a publication. Therefore, if the chromatographer seeks to duplicate the published values, the column oven must be operated at the same temperature.

Trials chromatographing pesticidal compounds and metabolites in this laboratory, using electron capture and flame photometric detection (phosphorus mode), have shown conclusively that a linear relationship exists between column temperature and RRT over a temperature range of 170 to 204°C. The RRT of a given compound eluting later than the reference compound varies inversely with the increase in temperature, whereas the values of compounds eluting earlier than the reference compound generally vary in direct proportion to the temperature increase.

In view of these findings, the development of tables of RRT values applicable at any given temperature in the range of 170 to 204°C provides the pesticide chemist with precise and useful data, irrespective of the oven pyrometer readout at any time.

METHOD

Apparatus and Reagents

(a) *Gas chromatographic equipment*.—Tracor Model 220 gas chromatograph equipped with parallel plate, tritium source electron capture detector; Melpar flame photometric detector with 526 nm filter; oven accommodating up to 4 U-shaped columns, each 1.829 m (6') × 4 mm id; Vycor glass injection port "demister" tube for off-column injections.

(b) *Computer*.—Hewlett-Packard Model 2100A equipped with teleprinter readout.

(c) *Thermometers*.—Mercury, precision, -1 to 201°C, in 0.2° graduations, 610 mm long, National Bureau of Standards (NBS) calibrated; mercury, 0-250°C, graduated in 1° subdivisions, accuracy ±0.5°C.

(d) *Standards*.—Standard pesticide mixtures of

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up to 13 compounds, none of which would produce peak overlaps with any other component compound. Appropriate dilutions made with isooctane.

(e) *Column conditioner*.—Silyl 8, 1.0 ml ampoules (Pierce Chemical Co., Rockford, IL 61105).

Column Packings

All column packing materials were purchased precoated, on quality specifications, from commercial suppliers. The materials coated on Chromosorb W supports were purchased from Supelco, Inc., Bellefonte, PA 16823, and those coated on Gas-Chrom Q from Applied Science Laboratories, Inc., State College, PA 16801.

The GLC columns selected for the development of these data were as follows:

(1) Via electron capture detection:

5% OV-210 on 100-120 mesh Chromosorb W (HP)

10% DC-200 on 80-100 mesh Chromosorb W (HP)

4% SE-30/6% QF-1 on 80-100 mesh Chromosorb W (HP)

1.5% OV-17/1.95% QF-1 on 100-120 mesh Chromosorb W (HP)

5% DC-200/7.5% QF-1 on 80-100 mesh Chromosorb W (HP)

1.8% OV-17/6.4% OV-210 on 80-100 mesh Gas-Chrom Q

(2) Via flame photometric detection:

10% OV-210 on 100-120 mesh Gas-Chrom Q

4% SE-30/6% OV-210 on 80-100 mesh Gas-Chrom Q

1.5% OV-17/1.95% OV-210 on 100-120 mesh Gas-Chrom Q

Column Preparation and Evaluation

Each column, after it was packed and the ends were plugged with silanized glass wool, was heat-conditioned by placing it in the GLC oven with the inlet end connected to the injection port and with the outlet end vented inside the oven. Conditioning was carried out at 245°C for 72-96 hr with a 60 ml carrier gas (nitrogen)/ml flow through the column.

Following the heat-conditioning period, the oven temperature was reduced to the operating level. Each column to be used for chlorinated compounds (electron capture detection) received 3 consecutive injections of 25 μ l each of Silyl 8. The injections were spaced ca 30 min apart. The effluent was vented into the oven for ≥ 3 hr after the final injection and before the outlet end of the column was connected to the detector transfer line.

Each of the columns of mixed liquid phases was carefully evaluated to be certain that the stated

proportions of liquid phase materials were correct. This was carried out by adjusting the oven temperature to precisely $200 \pm 0.5^\circ\text{C}$ and chromatographing a mixture of 13 chlorinated pesticides. Using the retention of aldrin as the reference point, the RRT_A was computed for all other compounds. From past research in this laboratory (4), the correct RRT_A values for all compounds at the given temperature were known so that values obtained on the new columns could be compared.

Adjustment and Calibration of Column Temperature

To ensure maximum validity, it was necessary to monitor column oven temperature more accurately than is often possible with the built-in pyrometer. A long-stem, NBS-calibrated glass thermometer was inserted into one of the unused injection ports and was lowered until the mercury bulb was at the center location of the columns. The oven temperature was adjusted to precisely $200 \pm 0.2^\circ\text{C}$. This was held ca 3 hr to ensure equilibration. The thermometer was then raised until the mercury bulb reached the same vertical point, ca 5" from the top of the oven, where the bulb of a lower quality glass thermometer would be placed throughout the study. The temperature differential never exceeded 0.4°C , with a higher reading being recorded near the top of the oven, presumably due to heat radiated from the 225°C injection block. The lower quality thermometer, previously calibrated against the NBS one, was then inserted and the temperature reading was logged. Any subsequent oven temperature adjustment required during this portion of the study was targeted to the initial reading of the thermometer used as the continuous monitor.

Upon completion of the foregoing calibration, the instrument pyrometer was adjusted to coincide with the established temperature so that both pyrometer and mercury thermometer were reading identically.

Discussion

In the development of the data for Tables 1-9,² 18 RRT values are given for each compound at 2°C intervals. Obviously, the task of determining RRT values at each of the 18 temperature intervals would be overwhelming. It was recognized from previously obtained data, using both electron capture and flame photometric detectors, that a linear relationship existed between column temperature and RRT . Figure 1

² In Tables 1-9, ME = methyl esters, IPE = isopropyl esters, and BE = butyl esters.

Table 1. Retention ratios, relative to aldrin, of 51 pesticides and metabolites on a column of 5% OV-210 on 100-120 mesh Chromosorb W (HP) at temperatures from 170 to 204°C; electron capture detector, tritium source, parallel plate; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 50 ml/min

	170	174	178	182	186	190	194	198	202	204	
				↓							
0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	Mevinphos
0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	2,4-D (ME)
0.93	0.94	0.94	0.94	0.94	0.94	0.95	0.95	0.95	0.95	0.95	Phorate
0.92	0.92	0.92	0.93	0.93	0.94	0.95	0.95	0.95	0.95	0.95	α -BHC
0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89	CDEC
0.88	0.87	0.87	0.87	0.88	0.88	0.88	0.88	0.88	0.88	0.88	2,4-D (IPE)
0.87	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	Simazine
0.88	0.88	0.87	0.87	0.88	0.88	0.88	0.88	0.88	0.88	0.88	Atrazine
0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	Diazinon
0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	Lindane
1.08	1.08	1.07	1.07	1.06	1.06	1.04	1.03	1.03	1.01	1.01	2,4,5-T (ME)
0.97	0.97	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	β -BHC
1.30	1.29	1.29	1.27	1.26	1.25	1.23	1.22	1.21	1.19	1.18	2,4-D (BE) I
1.07	1.07	1.06	1.06	1.06	1.06	1.04	1.04	1.03	1.03	1.02	δ -BHC
0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.88	0.88	Heptachlor
1.36	1.34	1.33	1.32	1.30	1.29	1.27	1.26	1.25	1.23	1.22	2,4,5-T (IPE)
1.47	1.46	1.44	1.43	1.41	1.40	1.38	1.36	1.35	1.33	1.32	2,4-D (BE) II
1.51	1.51	1.50	1.49	1.48	1.48	1.47	1.46	1.45	1.44	1.43	Dichloro
2.18	2.15	2.12	2.08	2.06	2.04	2.01	1.98	1.92	1.89	1.86	Dimethoate
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Aldrin (REFERENCE)
1.41	1.40	1.39	1.38	1.37	1.36	1.35	1.34	1.33	1.32	1.31	Ronnel
1.43	1.42	1.41	1.40	1.39	1.38	1.37	1.36	1.35	1.34	1.33	1-Hydroxychloridene
3.17	3.12	3.07	3.02	2.97	2.92	2.86	2.83	2.79	2.73	2.68	Methyl parathion
2.02	2.00	1.98	1.97	1.95	1.93	1.91	1.89	1.87	1.85	1.83	Heptachlor epoxide
3.20	3.14	3.08	3.02	2.96	2.91	2.85	2.79	2.73	2.67	2.61	Malathion
2.84	2.80	2.75	2.71	2.67	2.63	2.59	2.54	2.50	2.46	2.41	DCPA
2.06	2.04	2.02	1.99	1.97	1.94	1.91	1.89	1.87	1.84	1.81	Dyrene
1.87	1.86	1.83	1.82	1.80	1.79	1.77	1.75	1.73	1.71	1.69	<i>o,p'</i> -DDE
1.97	1.96	1.93	1.90	1.88	1.86	1.84	1.82	1.80	1.78	1.75	Chlorobenzide
4.24	4.17	4.09	4.02	3.94	3.87	3.78	3.71	3.63	3.56	3.48	Ethyl parathion
2.63	2.60	2.57	2.54	2.51	2.48	2.45	2.42	2.39	2.36	2.33	Endosulfan I
2.24	2.21	2.18	2.15	2.12	2.10	2.07	2.04	2.01	1.98	1.95	<i>p,p'</i> -DDE
3.10	3.05	3.00	2.94	2.89	2.84	2.79	2.74	2.69	2.64	2.59	DDA (ME)
4.47	4.40	4.32	4.24	4.17	4.09	4.01	3.93	3.85	3.77	3.69	Captan
4.04	3.96	3.92	3.86	3.78	3.72	3.65	3.59	3.53	3.46	3.39	Folpet
3.21	3.17	3.13	3.09	3.04	3.00	2.96	2.92	2.88	2.83	2.79	Dieldrin
2.07	2.04	2.01	1.98	1.96	1.92	1.89	1.86	1.83	1.80	1.77	Parthane
2.74	2.70	2.67	2.63	2.59	2.55	2.51	2.47	2.43	2.39	2.35	<i>o,p'</i> -DDD
2.82	2.87	2.83	2.79	2.74	2.70	2.65	2.61	2.57	2.52	2.48	<i>o,p'</i> -DDT
3.82	3.77	3.72	3.67	3.61	3.56	3.50	3.45	3.39	3.34	3.29	Endrin
2.78	2.73	2.70	2.67	2.64	2.61	2.58	2.55	2.52	2.49	2.46	Chlordecone
4.10	4.03	3.96	3.89	3.82	3.75	3.68	3.61	3.53	3.46	3.38	<i>p,p'</i> -DDD
4.88	4.91	4.83	4.75	4.67	4.59	4.51	4.43	4.35	4.27	4.19	Endosulfan II
6.0	5.99	5.76	5.64	5.51	5.37	5.25	5.12	4.99	4.86	4.73	Ethion
4.47	4.39	4.31	4.23	4.15	4.07	3.98	3.90	3.82	3.74	3.66	<i>p,p'</i> -DDT
5.29	5.19	5.08	4.99	4.89	4.79	4.69	4.58	4.48	4.37	4.27	Carbophenothion
12.3	12.0	11.7	11.4	11.1	10.8	10.4	10.1	9.8	9.5	9.2	Dilan I
4.08	4.01	3.95	3.89	3.84	3.78	3.73	3.68	3.62	3.57	3.52	Mirex
7.4	7.2	7.0	6.8	6.7	6.5	6.3	6.1	5.94	5.76	5.59	Methoxychlor
14.2	13.8	13.4	13.0	12.6	12.3	11.9	11.5	11.1	10.7	10.3	Dilan II
21.1	20.9	20.0	19.5	18.9	18.4	17.9	17.3	16.7	16.2	15.6	Tetradifon
24.7	24.0	23.3	22.6	21.9	21.2	20.5	19.8	19.2	18.5	17.8	Azinphosmethyl
	170	174	178	182	186	190	194	198	202	204	

Table 2. Retention ratios, relative to aldrin, of 51 pesticides and metabolites on a column of 10% DC-200 on 80-100 mesh Chromosorb W (HP) at temperatures from 170 to 204°C; electron capture detector, tritium source, parallel plate; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 120 ml/min

	170	174	178	182	186	190	194	198	202	204	
	0.16	0.18	0.18	0.17	0.17	0.17	0.18	0.18	0.19	0.19	Mevinphos
	0.27	0.28	0.28	0.29	0.29	0.30	0.30	0.30	0.31	0.31	2,4-D (ME)
	0.34	0.34	0.35	0.35	0.36	0.36	0.37	0.37	0.38	0.38	Phorate
	0.35	0.35	0.36	0.36	0.37	0.37	0.38	0.38	0.39	0.40	α -BHC
	0.36	0.36	0.36	0.36	0.37	0.37	0.38	0.38	0.39	0.40	CDEC
	0.38	0.38	0.38	0.39	0.39	0.40	0.40	0.41	0.41	0.41	2,4-D (IPE)
	0.39	0.39	0.39	0.40	0.40	0.40	0.41	0.41	0.41	0.41	Simazine
	0.41	0.41	0.41	0.42	0.42	0.42	0.43	0.43	0.43	0.44	Atrazine
	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52	Diazinon
	0.43	0.43	0.44	0.44	0.45	0.45	0.46	0.46	0.46	0.50	Lindane
	0.48	0.48	0.48	0.48	0.49	0.49	0.49	0.49	0.50	0.50	2,4,5-T (ME)
	0.38	0.38	0.38	0.39	0.40	0.40	0.41	0.41	0.42	0.43	β -BHC
	0.50	0.50	0.50	0.50	0.51	0.51	0.51	0.51	0.51	0.51	2,4-D (BE) I
	0.45	0.45	0.46	0.46	0.47	0.47	0.48	0.48	0.49	0.50	δ -BHC
	0.78	0.78	0.78	0.78	0.79	0.79	0.80	0.80	0.80	0.81	Heptachlor
	0.67	0.67	0.67	0.67	0.68	0.68	0.68	0.68	0.68	0.68	2,4,5-T (IPE)
	0.71	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.69	0.69	2,4-D (BE) II
	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.53	0.53	Dichloro
	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.46	0.46	Dimethoate
	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Aldrin (REFERENCE)
	0.82	0.81	0.81	0.81	0.80	0.80	0.80	0.80	0.79	0.79	Ronnel
	1.01	1.01	1.01	1.01	1.00	1.00	1.00	1.00	0.99	0.99	1-Hydroxychlorodene
	0.68	0.68	0.68	0.68	0.69	0.69	0.69	0.69	0.69	0.69	Methyl parathion
	1.29	1.29	1.28	1.28	1.27	1.26	1.26	1.24	1.23	1.22	Heptachlor epoxide
	0.77	0.78	0.78	0.78	0.79	0.79	0.79	0.80	0.80	0.81	Malathion
	1.10	1.09	1.09	1.08	1.08	1.08	1.08	1.04	1.03	1.02	DCPA
	1.29	1.28	1.27	1.26	1.25	1.24	1.23	1.22	1.21	1.20	Dyrene
	1.65	1.64	1.62	1.61	1.59	1.58	1.57	1.55	1.54	1.53	<i>o,p'</i> -DDE
	1.43	1.42	1.41	1.40	1.39	1.38	1.37	1.36	1.35	1.34	Chlorobenzide
	1.03	1.02	1.01	1.00	0.99	0.99	0.98	0.97	0.96	0.95	Ethyl parathion
	1.64	1.63	1.62	1.61	1.60	1.59	1.58	1.56	1.55	1.54	Endosulfan I
	2.09	2.07	2.06	2.05	2.00	1.98	1.96	1.92	1.90	1.88	<i>p,p'</i> -DDE
	1.81	1.79	1.77	1.75	1.73	1.71	1.69	1.67	1.65	1.63	DDA (ME)
	1.21	1.21	1.21	1.20	1.19	1.19	1.18	1.17	1.17	1.16	Captan
	1.30	1.29	1.29	1.28	1.27	1.26	1.25	1.24	1.23	1.23	Folpet
	1.99	1.98	1.96	1.95	1.93	1.92	1.90	1.89	1.87	1.86	Dieldrin
	2.80	2.86	2.83	2.80	2.78	2.76	2.74	2.72	2.69	2.68	Perthane
	2.13	2.11	2.09	2.08	2.04	2.01	1.99	1.97	1.94	1.92	<i>o,p'</i> -DDD
	2.80	2.88	2.83	2.79	2.76	2.72	2.68	2.65	2.61	2.58	<i>o,p'</i> -DDT
	2.22	2.20	2.19	2.17	2.16	2.14	2.13	2.11	2.09	2.08	Endrin
	3.07	3.03	3.00	2.98	2.93	2.90	2.86	2.83	2.79	2.76	Chlordecone
	2.72	2.69	2.66	2.62	2.59	2.56	2.52	2.49	2.45	2.42	<i>p,p'</i> -DDD
	2.29	2.27	2.25	2.24	2.22	2.20	2.18	2.17	2.15	2.13	Endosulfan II
	3.04	3.00	2.95	2.91	2.88	2.81	2.77	2.72	2.68	2.63	Ethion
	3.72	3.67	3.62	3.57	3.51	3.46	3.40	3.35	3.29	3.24	<i>p,p'</i> -DDT
	3.42	3.37	3.32	3.26	3.21	3.16	3.11	3.06	3.01	2.96	Carbophenothion
	3.23	3.19	3.15	3.11	3.06	3.02	2.98	2.93	2.89	2.85	Dilan I
	6.9	6.8	6.7	6.6	6.5	6.4	6.3	6.2	6.1	6.0	Mirex
	6.1	6.0	5.89	5.77	5.68	5.54	5.42	5.30	5.18	5.06	Methoxychlor
	4.07	4.02	3.98	3.89	3.83	3.77	3.70	3.64	3.58	3.52	Dilan II
	6.4	6.3	6.2	6.1	5.97	5.85	5.73	5.62	5.50	5.38	Tetradifon
	6.4	6.3	6.2	6.0	5.92	5.81	5.69	5.58	5.46	5.34	Azinphosmethyl
	170	174	178	182	186	190	194	198	202	204	

Table 3. Retention ratios, relative to aldrin, of 51 pesticides and metabolites on a column of 1.5% OV-17/1.95% QF-1 on 100-120 mesh Chromosorb W (HP) at temperatures from 170 to 204°C; electron capture detector, tritium source, parallel plate; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 60 ml/min

	170	174	178	182	186	190	194	198	202	204	
	0.32	0.32	0.32	0.32	0.32	0.32	0.33	0.33	0.33	0.33	Mevinphos
	0.44	0.45	0.45	0.45	0.45	0.46	0.46	0.46	0.47	0.47	2,4-D (ME)
	0.48	0.48	0.48	0.48	0.48	0.50	0.50	0.51	0.51	0.52	Phorate
	0.48	0.48	0.48	0.50	0.50	0.51	0.51	0.52	0.52	0.53	<i>n</i> -BHC
	0.54	0.54	0.54	0.55	0.55	0.55	0.55	0.55	0.56	0.56	CDEC
	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	2,4-D (IPE)
	0.70	0.70	0.70	0.69	0.69	0.69	0.69	0.69	0.67	0.67	Simazine
	0.69	0.69	0.69	0.69	0.69	0.69	0.67	0.67	0.67	0.67	Atrazine
	0.67	0.67	0.66	0.66	0.66	0.66	0.65	0.65	0.64	0.64	Diazinon
	0.66	0.67	0.67	0.67	0.67	0.66	0.66	0.66	0.66	0.66	Lindane
	0.76	0.76	0.76	0.76	0.76	0.76	0.74	0.74	0.73	0.73	2,4,5-T (ME)
	0.82	0.82	0.82	0.81	0.81	0.81	0.81	0.80	0.80	0.80	β -BHC
	0.86	0.86	0.84	0.84	0.83	0.82	0.81	0.80	0.80	0.79	2,4-D (BE) I
	0.97	0.97	0.96	0.96	0.95	0.94	0.94	0.93	0.92	0.92	δ -BHC
	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	Heptachlor
	0.84	0.84	0.83	0.82	0.81	0.80	0.80	0.80	0.80	0.80	2,4,5-T (IPE)
	1.03	1.02	1.01	1.01	1.00	0.99	0.98	0.96	0.94	0.93	2,4-D (BE) II
	1.02	1.02	1.01	1.01	1.01	1.00	1.00	1.00	0.99	0.99	Dichloro
	1.17	1.16	1.15	1.14	1.13	1.11	1.10	1.08	1.06	1.03	Dimethoate
	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Aldrin (REFERENCE)
	1.17	1.16	1.16	1.16	1.14	1.13	1.12	1.11	1.10	1.09	Ronnel
	1.41	1.40	1.39	1.38	1.36	1.34	1.33	1.31	1.29	1.27	1-Hydroxychlordene
	1.71	1.69	1.67	1.66	1.64	1.62	1.59	1.57	1.55	1.52	Methyl parathion
	1.70	1.69	1.68	1.67	1.66	1.64	1.63	1.62	1.61	1.59	Heptachlor epoxide
	2.07	2.04	2.01	1.98	1.95	1.92	1.89	1.87	1.84	1.81	Malathion
	1.82	1.80	1.78	1.76	1.74	1.72	1.70	1.68	1.66	1.64	DCPA
	2.15	2.13	2.11	2.09	2.07	2.05	2.02	2.00	1.98	1.96	Dyrene
	2.14	2.12	2.09	2.07	2.05	2.03	2.01	1.99	1.96	1.94	<i>o,p'</i> -DDE
	2.20	2.18	2.16	2.14	2.12	2.10	2.08	2.06	2.03	2.01	Chlorobenzide
	2.32	2.29	2.26	2.23	2.19	2.16	2.13	2.09	2.06	2.03	Ethyl parathion
	2.20	2.18	2.16	2.15	2.13	2.11	2.10	2.08	2.06	2.03	Endosulfan I
	2.75	2.72	2.68	2.64	2.61	2.58	2.54	2.51	2.47	2.43	<i>p,p'</i> -DDE
	2.97	2.93	2.89	2.84	2.79	2.75	2.71	2.66	2.62	2.57	DDA (ME)
	3.27	3.22	3.18	3.13	3.07	3.04	3.00	2.96	2.91	2.86	Captan
	3.32	3.27	3.23	3.18	3.13	3.08	3.04	2.99	2.95	2.91	Folpet
	2.80	2.77	2.75	2.72	2.69	2.67	2.64	2.61	2.58	2.53	Dieldrin
	3.52	3.46	3.41	3.36	3.30	3.24	3.19	3.14	3.08	3.03	Perthane
	3.34	3.29	3.25	3.20	3.15	3.11	3.06	3.01	2.97	2.92	<i>o,p'</i> -DDD
	3.89	3.84	3.80	3.73	3.71	3.68	3.60	3.54	3.48	3.43	<i>o,p'</i> -DDT
	3.47	3.43	3.40	3.36	3.33	3.29	3.26	3.22	3.18	3.15	Endrin
	3.26	3.23	3.19	3.16	3.13	3.09	3.05	3.00	2.96	2.93	Chlordecone
	4.86	4.87	4.89	4.81	4.73	4.65	4.58	4.50	4.42	4.34	<i>p,p'</i> -DDD
	4.48	4.39	4.34	4.28	4.23	4.17	4.11	4.05	3.99	3.94	Endosulfan II
	6.1	5.97	5.85	5.73	5.61	5.49	5.36	5.24	5.12	5.00	Ethion
	6.57	6.48	6.39	6.29	6.20	6.11	6.01	5.92	5.83	5.74	<i>p,p'</i> -DDT
	6.4	6.2	6.1	5.99	5.88	5.76	5.64	5.52	5.40	5.28	Carbophenothion
	8.0	7.9	7.7	7.5	7.4	7.2	7.1	6.9	6.7	6.6	Dilan I
	7.7	7.6	7.5	7.3	7.2	7.1	7.0	6.9	6.8	6.7	Mirex
	12.4	12.1	11.8	11.5	11.3	11.0	10.7	10.4	10.1	9.8	Methoxychlor
	8.4	8.2	8.0	7.8	7.6	7.4	7.2	7.0	6.9	6.7	Dilan II
	16.9	16.5	16.1	15.7	15.3	14.9	14.5	14.1	13.7	13.3	Tetradifon
	24.0	23.4	22.8	22.2	21.6	21.0	20.4	19.8	19.2	18.6	Azinphosmethyl
	170	174	178	182	186	190	194	198	202	204	

Table 4. Retention ratios, relative to aldrin, of 51 pesticides and metabolites on a column of 4% SE-30/6% QF-1 on 80-100 mesh Chromosorb W (HP) at temperatures from 170 to 204°C; electron capture detector, tritium source, parallel plate; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 70 ml/min

	170	174	178	182	186	190	194	198	202	204	
0.27	0.28	0.28	0.28	0.29	0.29	0.30	0.30	0.30	0.31	0.31	Mevinphos
0.39	0.39	0.39	0.40	0.40	0.40	0.41	0.41	0.42	0.42	0.43	2,4-D (ME)
0.43	0.43	0.43	0.44	0.44	0.44	0.45	0.45	0.46	0.46	0.46	Phorate
0.42	0.43	0.43	0.44	0.44	0.44	0.45	0.45	0.46	0.47	0.47	n-BHC
0.44	0.44	0.45	0.45	0.46	0.46	0.47	0.47	0.48	0.48	0.49	CDEC
0.54	0.54	0.54	0.54	0.55	0.55	0.55	0.55	0.55	0.55	0.55	2,4-D (IPE)
0.52	0.52	0.53	0.53	0.53	0.53	0.53	0.53	0.54	0.54	0.54	Simazine
0.55	0.55	0.55	0.55	0.55	0.55	0.56	0.56	0.57	0.57	0.57	Atrazine
0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	Diazinon
0.54	0.54	0.55	0.55	0.55	0.56	0.57	0.57	0.58	0.59	0.59	Lindane
0.66	0.66	0.66	0.66	0.66	0.66	0.67	0.67	0.68	0.68	0.68	2,4-D (BE) I
0.57	0.57	0.58	0.58	0.58	0.59	0.59	0.59	0.60	0.60	0.61	β-BHC
0.65	0.64	0.64	0.63	0.63	0.62	0.62	0.61	0.60	0.59	0.58	2,4-D (BE) II
0.65	0.65	0.66	0.66	0.66	0.67	0.67	0.67	0.68	0.68	0.69	δ-BHC
0.60	0.60	0.61	0.61	0.61	0.61	0.62	0.62	0.62	0.63	0.63	Heptachlor
0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69	2,4,5-T (IPE)
0.66	0.67	0.66	0.66	0.66	0.66	0.67	0.67	0.68	0.68	0.68	2,4-D (BE) II
0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.83	0.83	0.83	Dichloro
0.96	0.95	0.94	0.94	0.93	0.93	0.92	0.91	0.90	0.89	0.88	Dimethoate
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Aldrin (REFERENCE)
1.01	1.00	1.00	0.99	0.98	0.98	0.97	0.96	0.95	0.94	0.93	Ronnel
1.04	1.04	1.04	1.03	1.03	1.03	1.03	1.03	1.03	1.02	1.02	1-Hydroxychlorodene
1.49	1.48	1.47	1.46	1.45	1.44	1.43	1.42	1.41	1.40	1.39	Methyl parathion
1.53	1.53	1.52	1.51	1.50	1.49	1.48	1.47	1.46	1.45	1.44	Heptachlor epoxide
1.70	1.68	1.66	1.64	1.63	1.61	1.59	1.57	1.55	1.53	1.51	Malathion
1.84	1.83	1.81	1.80	1.79	1.78	1.76	1.74	1.72	1.70	1.68	DCPA
1.93	1.92	1.91	1.90	1.89	1.88	1.86	1.84	1.82	1.80	1.78	Dyrene
1.87	1.86	1.85	1.83	1.82	1.80	1.79	1.77	1.75	1.73	1.71	o,p'-DDE
1.82	1.81	1.80	1.79	1.78	1.76	1.74	1.72	1.70	1.68	1.66	Chlorbenside
2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	Ethyl parathion
1.99	1.98	1.97	1.96	1.94	1.93	1.91	1.89	1.87	1.85	1.83	Endosulfan I
2.16	2.14	2.11	2.09	2.07	2.05	2.02	2.00	1.98	1.95	1.93	p,p'-DDE
2.27	2.25	2.22	2.19	2.16	2.13	2.10	2.07	2.04	2.01	1.98	DDA (ME)
2.27	2.25	2.23	2.21	2.19	2.16	2.14	2.12	2.10	2.07	2.04	Captan
2.22	2.20	2.18	2.16	2.14	2.12	2.10	2.08	2.06	2.04	2.02	Folpet
2.43	2.41	2.39	2.37	2.35	2.33	2.31	2.29	2.27	2.25	2.23	Dieldrin
2.43	2.40	2.37	2.33	2.30	2.27	2.24	2.20	2.17	2.14	2.11	Perthane
2.34	2.31	2.29	2.27	2.24	2.22	2.19	2.17	2.15	2.12	2.10	o,p'-DDD
3.02	2.97	2.93	2.89	2.84	2.80	2.76	2.73	2.69	2.64	2.58	o,p'-DDT
2.76	2.73	2.71	2.69	2.67	2.64	2.62	2.60	2.58	2.53	2.51	Endrin
2.97	2.94	2.91	2.89	2.86	2.83	2.80	2.78	2.75	2.72	2.69	Chlordecone
3.22	3.17	3.13	3.08	3.04	2.99	2.94	2.90	2.86	2.82	2.77	p,p'-DDD
3.19	3.16	3.13	3.10	3.07	3.04	3.00	2.97	2.94	2.91	2.88	Endosulfan II
4.08	4.02	3.96	3.89	3.81	3.76	3.68	3.60	3.53	3.47	3.40	Ethion
4.04	3.98	3.92	3.86	3.80	3.73	3.67	3.61	3.54	3.48	3.43	p,p'-DDT
4.06	4.02	3.96	3.90	3.83	3.78	3.72	3.66	3.59	3.52	3.47	Carbophenothion
6.2	6.1	5.98	5.83	5.72	5.60	5.49	5.38	5.27	5.17	5.02	Dilan I
6.1	6.0	5.96	5.87	5.78	5.68	5.60	5.52	5.43	5.33	5.24	Mirex
6.7	6.5	6.4	6.3	6.1	5.98	5.84	5.70	5.57	5.42	5.29	Methoxychlor
7.4	7.2	7.1	6.9	6.8	6.6	6.5	6.3	6.2	6.0	5.87	Dilan II
11.8	11.3	11.1	10.8	10.6	10.3	10.1	9.8	9.6	9.3	9.0	Tetradifon
12.5	12.2	11.9	11.7	11.4	11.1	10.8	10.5	10.0	9.7	9.4	Azinphosmethyl

Column operating temperature with carrier flow at 120 ml/min																				
170	174	178	182	186	190	194	198	202	204											
0.29	0.29	0.29	0.29	0.30	0.30	0.30	0.30	0.30	0.30	0.31	Mevinphos									
0.40	0.40	0.40	0.40	0.40	0.41	0.41	0.41	0.41	0.41	0.42	2,4-D (ME)									
0.43	0.44	0.44	0.44	0.44	0.44	0.45	0.45	0.45	0.45	0.46	Phorate									
0.43	0.43	0.44	0.44	0.44	0.45	0.45	0.45	0.46	0.46	0.46	n-BHC									
0.45	0.45	0.45	0.46	0.46	0.46	0.47	0.47	0.47	0.47	0.48	CDEC									
0.54	0.54	0.54	0.54	0.54	0.55	0.55	0.55	0.55	0.55	0.56	2,4-D (IPE)									
0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	Simazine									
0.55	0.55	0.55	0.55	0.56	0.56	0.56	0.56	0.56	0.57	0.57	Atrazine									
0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	Diazinon									
0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	Lindane									
0.57	0.57	0.57	0.57	0.58	0.58	0.58	0.58	0.58	0.58	0.58	2,4,5-T (ME)									
0.57	0.58	0.58	0.58	0.58	0.59	0.59	0.59	0.59	0.59	0.59	β-BHC									
0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	2,4-D (BE I)									
0.55	0.55	0.55	0.55	0.56	0.56	0.56	0.56	0.56	0.56	0.56	5-BHC									
0.50	0.50	0.50	0.50	0.51	0.51	0.51	0.51	0.51	0.51	0.51	Heptachlor									
0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	2,4,5-T (IPE)									
0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	2,4-D (BE II)									
0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	Dichloro									
0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	Dimethoate									
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Aldrin (REFERENCE)									
1.02	1.01	1.01	1.01	1.00	1.00	0.99	0.99	0.98	0.98	0.98	Ronnel									
1.27	1.27	1.25	1.24	1.23	1.22	1.21	1.20	1.19	1.18	1.16	1-Hydroxychlorodene									
1.34	1.32	1.31	1.31	1.40	1.40	1.47	1.46	1.45	1.43	1.42	Methyl parathion									
1.34	1.33	1.33	1.32	1.31	1.31	1.40	1.40	1.40	1.40	1.40	Heptachlor epoxide									
1.73	1.71	1.69	1.67	1.65	1.63	1.61	1.59	1.57	1.55	1.53	Malathion									
1.70	1.68	1.67	1.65	1.63	1.62	1.60	1.58	1.57	1.55	1.54	DCPA									
1.34	1.33	1.31	1.30	1.40	1.40	1.46	1.46	1.44	1.43	1.41	Dyrene									
1.86	1.86	1.83	1.82	1.81	1.80	1.80	1.80	1.80	1.80	1.80	o,p'-DDE									
1.82	1.81	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	Chlorbensene									
2.14	2.11	2.09	2.07	2.05	2.03	2.01	1.99	1.97	1.95	1.93	Ethyl parathion									
2.00	1.99	1.97	1.96	1.96	1.94	1.93	1.91	1.89	1.88	1.87	Endosulfan I									
2.16	2.13	2.11	2.09	2.07	2.05	2.03	2.01	1.99	1.97	1.95	p,p'-DDE									
2.26	2.25	2.22	2.19	2.16	2.13	2.10	2.07	2.04	2.01	1.98	DDA (ME)									
2.35	2.33	2.31	2.29	2.26	2.23	2.21	2.19	2.17	2.14	2.12	Captan									
2.24	2.23	2.21	2.19	2.17	2.15	2.13	2.12	2.10	2.08	2.06	Folpet									

Table 6. Retention ratios, relative to aldrin, of 51 pesticides and metabolites on a column of 1.6% OV-17/6.4% OV-210 on 80-100 mesh Chromosorb W (HP) at temperatures from 170 to 204°C; electron capture detector, tritium source, parallel plate; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 70 ml/min

170	174	178	182	186	190	194	198	202	
0.36	0.37	0.37	0.37	0.37	0.38	0.38	0.38	0.39	Mevinphos
0.48	0.48	0.48	0.48	0.49	0.49	0.50	0.50	0.51	2,4-D (ME)
0.51	0.51	0.51	0.51	0.52	0.52	0.53	0.53	0.54	Phorate
0.49	0.50	0.50	0.51	0.52	0.52	0.53	0.54	0.55	n-BHC
0.56	0.56	0.56	0.57	0.57	0.57	0.58	0.58	0.59	CDEC
0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62	2,4-D (IPE)
0.72	0.72	0.71	0.71	0.71	0.71	0.71	0.70	0.70	Simazine
0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	Atrazine
0.71	0.70	0.70	0.69	0.69	0.68	0.67	0.67	0.66	Diazinon
0.69	0.69	0.69	0.69	0.70	0.70	0.70	0.70	0.71	Lindane
0.84	0.84	0.83	0.83	0.82	0.82	0.81	0.81	0.80	2,4,5-T (ME)
0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	β-BHC
0.96	0.96	0.95	0.95	0.94	0.93	0.92	0.91	0.91	2,4-D (BE) I
0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	α-BHC
0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	Heptachlor
1.02	1.02	1.01	1.01	1.00	0.99	0.99	0.98	0.96	2,4,5-T (IPE)
1.13	1.13	1.12	1.11	1.10	1.09	1.08	1.06	1.05	2,4-D (BE) II
1.14	1.14	1.14	1.14	1.13	1.13	1.13	1.13	1.12	Dichloro
1.30	1.29	1.29	1.28	1.27	1.26	1.25	1.25	1.24	Dimethoate
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Aldrin (REFERENCE)
1.24	1.23	1.22	1.21	1.20	1.19	1.18	1.17	1.16	Ronnel
1.43	1.42	1.41	1.41	1.40	1.39	1.38	1.37	1.36	1-Hydroxychloro
2.02	2.01	1.99	1.98	1.96	1.95	1.93	1.92	1.90	Methyl parathion
1.81	1.79	1.78	1.77	1.76	1.75	1.73	1.72	1.71	Heptachlor epoxide
2.37	2.33	2.30	2.26	2.22	2.18	2.15	2.11	2.07	Malathion
2.06	2.04	2.02	1.99	1.97	1.95	1.93	1.91	1.88	DCPA
2.14	2.12	2.09	2.07	2.04	2.02	2.00	1.97	1.95	Dyrene
2.04	2.02	2.00	1.98	1.95	1.93	1.91	1.89	1.87	o,p'-DDE
2.18	2.16	2.14	2.11	2.09	2.07	2.04	2.02	2.00	Chlorobenzide
2.80	2.76	2.73	2.69	2.66	2.62	2.59	2.55	2.52	Ethyl parathion
2.33	2.31	2.29	2.27	2.25	2.23	2.21	2.19	2.18	Endosulfan I
2.67	2.64	2.60	2.57	2.53	2.49	2.46	2.42	2.39	p,p'-DDE
3.07	3.02	2.97	2.92	2.87	2.82	2.78	2.73	2.68	DDA (ME)
3.58	3.54	3.49	3.44	3.40	3.35	3.30	3.26	3.21	Captan
3.51	3.46	3.42	3.37	3.33	3.28	3.24	3.20	3.15	Folpet
2.94	2.92	2.89	2.86	2.83	2.80	2.77	2.74	2.71	Dieldrin
3.24	3.18	3.12	3.07	3.01	2.95	2.90	2.84	2.78	Perthane
3.26	3.20	3.16	3.10	3.05	3.00	2.96	2.90	2.86	o,p'-DDD
3.81	3.75	3.70	3.64	3.58	3.52	3.46	3.40	3.35	o,p'-DDT
3.68	3.64	3.59	3.54	3.50	3.45	3.40	3.36	3.32	Endrin
3.18	3.15	3.12	3.09	3.06	3.03	3.00	2.97	2.94	Chlordecone
4.63	4.55	4.46	4.38	4.30	4.21	4.13	4.05	3.96	p,p'-DDD
4.60	4.54	4.48	4.42	4.35	4.30	4.24	4.17	4.12	Endosulfan II
6.2	6.0	5.92	5.79	5.67	5.54	5.42	5.29	5.16	Ethion
5.46	5.36	5.26	5.16	5.06	4.96	4.86	4.76	4.66	p,p'-DDT
6.35	6.2	6.1	5.96	5.83	5.69	5.56	5.43	5.30	Carbophenothion
9.1	8.9	8.7	8.5	8.3	8.2	8.0	7.8	7.6	Dilan I
7.0	6.9	6.8	6.7	6.6	6.5	6.4	6.3	6.2	Mirex
10.9	10.6	10.4	10.2	10.0	9.7	9.4	9.2	9.0	Methoxychlor
10.5	10.3	10.0	9.8	9.6	9.4	9.1	8.9	8.7	Dilan II
17.5	17.2	16.8	16.4	16.0	15.7	15.3	14.9	14.5	Tetradifon
24.5	23.8	23.2	22.5	21.9	21.3	20.7	20.0	19.4	Azinphosmethyl

Table 7. Retention ratios, relative to ethyl parathion, of 54 organophosphorus pesticides and metabolites on a column of 10% OV-210 on 100-120 mesh Gas-Chrom Q at temperatures from 170 to 204°C; flame photometric detector, 526 nm filter; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 70 ml/min

170	174	178	182	186	190	194	198	202	204	
0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.05	0.06	0.06	TEPP
0.06	0.06	0.06	0.07	0.07	0.07	0.08	0.08	0.08	0.09	Dichlorvos
0.12	0.12	0.12	0.13	0.13	0.13	0.13	0.13	0.14	0.14	Demeton thiono
0.14	0.14	0.15	0.15	0.15	0.16	0.16	0.17	0.17	0.18	Mevinphos
0.14	0.14	0.15	0.15	0.15	0.16	0.16	0.17	0.17	0.18	Thionazin
0.15	0.15	0.16	0.16	0.16	0.17	0.17	0.18	0.19	0.20	Phorate
0.16	0.16	0.17	0.17	0.17	0.18	0.18	0.19	0.20	0.21	Ethoprop
0.17	0.17	0.18	0.18	0.18	0.19	0.19	0.20	0.20	0.21	Diazinon
0.18	0.18	0.19	0.19	0.19	0.20	0.20	0.21	0.21	0.22	Sulfotep
0.19	0.19	0.20	0.21	0.21	0.22	0.22	0.23	0.24	0.25	Naled
0.04	0.04	0.04	0.05	0.05	0.05	0.06	0.06	0.06	0.07	Oxydemeton methyl
0.22	0.22	0.23	0.23	0.24	0.24	0.25	0.25	0.26	0.27	Disulfoton
0.25	0.25	0.26	0.26	0.27	0.27	0.28	0.28	0.29	0.30	Dioxathion
0.29	0.29	0.29	0.29	0.30	0.30	0.30	0.31	0.31	0.31	Demeton thiole
0.29	0.29	0.30	0.30	0.31	0.31	0.32	0.32	0.33	0.33	Dichlofenthion
0.33	0.33	0.33	0.34	0.34	0.34	0.35	0.35	0.35	0.36	Diazoxon
0.34	0.34	0.35	0.35	0.36	0.36	0.37	0.37	0.38	0.39	Ronnel
0.40	0.40	0.41	0.41	0.41	0.42	0.42	0.42	0.43	0.43	Chlorpyrifos
0.43	0.43	0.44	0.44	0.45	0.45	0.46	0.46	0.46	0.47	Zytron
0.47	0.47	0.47	0.47	0.48	0.48	0.48	0.49	0.49	0.49	Bromophos
0.48	0.48	0.48	0.49	0.49	0.49	0.50	0.50	0.50	0.51	Fenthion
0.47	0.47	0.48	0.48	0.49	0.49	0.50	0.51	0.51	0.52	Dimethoate
0.51	0.51	0.52	0.52	0.53	0.53	0.53	0.54	0.54	0.55	Ronoxon
0.53	0.53	0.54	0.54	0.55	0.55	0.56	0.57	0.57	0.58	Cyanox
0.59	0.59	0.59	0.60	0.60	0.60	0.60	0.61	0.61	0.61	Bromophos ethyl
0.67	0.67	0.66	0.66	0.66	0.65	0.65	0.65	0.64	0.64	Monocrotophos
0.75	0.75	0.74	0.74	0.73	0.73	0.72	0.71	0.71	0.70	Malathion
0.69	0.69	0.70	0.70	0.70	0.70	0.70	0.71	0.71	0.71	Iodofenphos
0.77	0.77	0.77	0.77	0.77	0.76	0.76	0.75	0.75	0.75	DEF
0.75	0.75	0.75	0.74	0.74	0.74	0.74	0.75	0.75	0.75	Phenthoate
0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	Fox
0.74	0.74	0.75	0.75	0.75	0.76	0.76	0.77	0.77	0.78	Methyl parathion
0.81	0.81	0.81	0.80	0.80	0.80	0.80	0.81	0.81	0.81	Schradan
0.84	0.84	0.84	0.85	0.85	0.85	0.85	0.86	0.86	0.86	Fenitrothion
1.02	1.01	1.01	0.99	0.99	0.98	0.97	0.96	0.94	0.93	Malaoxon
0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.96	0.96	0.96	Dicaphon
1.02	1.02	1.01	1.01	1.00	1.00	0.99	0.99	0.98	0.98	Crufomate
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Ethyl parathion (REFERENCE)
1.04	1.03	1.03	1.03	1.02	1.02	1.02	1.01	1.01	1.01	Methyl paraoxon
1.03	1.03	1.03	1.03	1.02	1.02	1.02	1.01	1.01	1.01	Amidithion
1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.12	1.12	1.12	Methidathion
1.22	1.22	1.21	1.20	1.19	1.19	1.18	1.17	1.16	1.15	Tetrachlorvinphos
1.27	1.27	1.26	1.25	1.25	1.24	1.24	1.23	1.22	1.21	Carbophenothion
1.38	1.37	1.36	1.35	1.34	1.33	1.32	1.31	1.30	1.29	Phosphamidon
1.42	1.41	1.40	1.39	1.37	1.37	1.36	1.35	1.34	1.33	Ethyl paraoxon
1.43	1.43	1.42	1.41	1.39	1.38	1.37	1.36	1.35	1.34	Ethion
1.64	1.63	1.62	1.61	1.60	1.59	1.58	1.57	1.56	1.55	Carbophenoxon
2.09	2.08	2.06	2.04	2.02	1.98	1.96	1.94	1.92	1.90	Phenkapton
4.26	4.21	4.16	4.11	4.06	3.97	3.92	3.87	3.82	3.77	Fensulfothion
4.37	4.32	4.27	4.22	4.18	4.13	4.08	4.03	3.98	3.93	EPN
4.65	4.59	4.54	4.48	4.43	4.37	4.32	4.26	4.21	4.15	Imidan
6.98	6.88	6.77	6.66	6.55	6.44	6.33	6.22	6.11	6.00	Azinphosmethyl
6.86	6.76	6.66	6.56	6.47	6.37	6.27	6.17	6.08	5.98	Famphur
7.01	6.90	6.79	6.69	6.58	6.47	6.36	6.25	6.14	6.03	Azinphosethyl
19.1	18.7	18.3	17.9	17.5	17.1	16.7	16.3	15.9	15.5	Coumaphos
170	174	178	182	186	190	194	198	202	204	

Table 2. Retention ratios, relative to ethyl parathion, of 54 organophosphorus pesticides and metabolites on a column of 1.5% OV-17/1.95% OV-210 on 100-120 mesh Gas-Chrom Q at temperatures from 170 to 204°C; flame photometric detector, 526 nm filter; all absolute retentions measured from injection point. Arrow indicates optimum column operating temperature with carrier flow at 70 ml/min

170	174	178	182	186	190	194	198	202	204	
0.04	0.04	0.05	0.05	0.05	0.06	0.06	0.06	0.07	0.07	TEPP
0.06	0.06	0.07	0.07	0.08	0.08	0.09	0.09	0.10	0.10	Dichlorvos
0.12	0.13	0.13	0.14	0.14	0.15	0.16	0.16	0.17	0.17	Mevinphos
0.16	0.16	0.17	0.17	0.18	0.18	0.19	0.19	0.20	0.20	Demeton thiono
0.19	0.19	0.20	0.20	0.21	0.21	0.22	0.22	0.23	0.23	Thionazin
0.20	0.20	0.21	0.21	0.22	0.22	0.23	0.23	0.24	0.24	Ethoprop
0.23	0.23	0.24	0.24	0.25	0.25	0.26	0.26	0.27	0.27	Phorate
0.24	0.25	0.25	0.25	0.26	0.26	0.27	0.27	0.28	0.28	Sulfotepp
0.26	0.27	0.27	0.28	0.28	0.29	0.29	0.30	0.30	0.31	Oxydemeton methyl
0.32	0.32	0.33	0.33	0.33	0.34	0.34	0.34	0.35	0.35	Diazinon
0.31	0.31	0.32	0.32	0.33	0.33	0.34	0.34	0.35	0.35	Naled
0.34	0.34	0.34	0.35	0.35	0.36	0.36	0.37	0.37	0.38	Demeton thiole
0.36	0.36	0.37	0.37	0.38	0.38	0.39	0.39	0.40	0.40	Disulfoton
0.37	0.38	0.38	0.39	0.39	0.40	0.40	0.41	0.41	0.42	Dioxathion
0.38	0.39	0.39	0.39	0.40	0.40	0.41	0.41	0.42	0.42	Diazoxon
0.44	0.44	0.45	0.45	0.46	0.46	0.47	0.47	0.48	0.48	Dichlorfenthion
0.51	0.51	0.51	0.52	0.53	0.53	0.54	0.54	0.55	0.55	Cyanox
0.53	0.53	0.53	0.54	0.54	0.55	0.55	0.56	0.56	0.57	Dimethoate
0.55	0.55	0.56	0.56	0.57	0.57	0.58	0.58	0.59	0.60	Ronnel
0.57	0.57	0.57	0.58	0.58	0.59	0.59	0.60	0.61	0.61	Ronnoxon
0.78	0.77	0.76	0.76	0.74	0.73	0.72	0.72	0.71	0.70	Monocrotophos
0.66	0.66	0.67	0.67	0.67	0.68	0.68	0.68	0.69	0.69	Zytron
0.74	0.74	0.74	0.75	0.75	0.75	0.75	0.75	0.76	0.76	Chlorpyrifos
0.74	0.74	0.74	0.75	0.75	0.76	0.76	0.77	0.78	0.78	Methyl parathion
0.79	0.79	0.79	0.79	0.79	0.80	0.80	0.80	0.80	0.81	Methyl paraoxon
0.93	0.93	0.92	0.92	0.91	0.91	0.90	0.89	0.89	0.88	Malaoxon
0.92	0.92	0.92	0.92	0.91	0.91	0.91	0.90	0.90	0.90	Malathion
0.85	0.85	0.86	0.86	0.86	0.87	0.87	0.87	0.88	0.88	Bromophos
0.90	0.90	0.90	0.90	0.91	0.91	0.91	0.91	0.91	0.90	Fenthion
0.90	0.90	0.90	0.90	0.91	0.91	0.91	0.91	0.91	0.92	Fenitrothion
1.02	1.01	1.01	1.00	1.00	0.99	0.99	0.98	0.98	0.97	Phosphamidon
1.25	1.23	1.21	1.19	1.18	1.16	1.14	1.13	1.11	1.09	Schradan
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Ethyl parathion (REFERENCE)
1.06	1.06	1.06	1.06	1.05	1.05	1.05	1.05	1.05	1.04	Ethyl paraoxon
1.05	1.05	1.05	1.05	1.06	1.06	1.06	1.06	1.05	1.05	Dicaphon
1.12	1.12	1.12	1.11	1.11	1.11	1.11	1.11	1.10	1.10	Bromophos ethyl
1.23	1.22	1.22	1.22	1.21	1.20	1.19	1.19	1.19	1.20	Amidithion
1.40	1.39	1.38	1.38	1.37	1.36	1.35	1.34	1.33	1.32	Crufomate
1.36	1.35	1.35	1.34	1.34	1.33	1.33	1.32	1.31	1.30	Phenthoate
1.51	1.50	1.49	1.49	1.48	1.47	1.46	1.45	1.44	1.43	Folex
1.51	1.50	1.49	1.49	1.48	1.47	1.46	1.45	1.44	1.44	DEF
1.57	1.57	1.56	1.56	1.55	1.54	1.54	1.53	1.53	1.52	Iodofenphos
1.72	1.71	1.70	1.69	1.68	1.67	1.66	1.65	1.65	1.64	Tetrachlorvinphos
1.74	1.73	1.73	1.72	1.72	1.71	1.71	1.70	1.70	1.69	Methidathion
2.69	2.67	2.64	2.62	2.59	2.57	2.54	2.52	2.49	2.46	Carbophenoxon
2.80	2.85	2.82	2.79	2.76	2.73	2.70	2.67	2.64	2.61	Ethion
2.99	2.97	2.94	2.92	2.89	2.87	2.84	2.82	2.79	2.77	Carbophenothion
4.65	4.60	4.56	4.51	4.46	4.42	4.37	4.32	4.27	4.23	Fensulfothion
5.57	5.50	5.42	5.35	5.27	5.20	5.12	5.05	4.97	4.90	Phenkapton
6.07	5.99	5.90	5.82	5.74	5.65	5.57	5.49	5.40	5.32	Famphur
6.63	6.53	6.44	6.34	6.25	6.16	6.06	5.97	5.88	5.78	EPN
7.95	7.84	7.72	7.61	7.50	7.38	7.27	7.15	7.04	6.93	Imidan
10.9	10.7	10.6	10.4	10.3	10.1	10.0	9.8	9.7	9.5	Azinphosmethyl
14.4	14.1	13.9	13.6	13.3	13.0	12.8	12.5	12.2	11.9	Azinphosethyl
22.2	21.8	21.3	20.9	20.4	20.0	19.5	19.1	18.6	18.2	Coumaphos

Table 3. Retention ratios, relative to parathion, of 54 organophosphorus pesticides and metabolites on a column of 4% SE-30/6% OV-210 on 80-100 mesh Gas-Chrom Q at temperatures from 170 to 204°C; flame photometric detector, 526 nm filter; all absolute retentions measured from injection point. Arrow indicates optimum operating temperature with carrier flow set at 75 ml/min

	170	174	178	182	186	190	194	198	202	204	
0.06	0.06	0.06	0.06	0.07	0.07	0.07	0.08	0.08	0.08	0.09	Dichlorvos
0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.06	0.06	0.06	0.07	TEPP
0.14	0.14	0.14	0.14	0.15	0.15	0.15	0.16	0.16	0.17	0.17	Mevinphos
0.15	0.15	0.15	0.16	0.16	0.16	0.17	0.17	0.18	0.19	0.19	Demeton thiono
0.16	0.16	0.16	0.17	0.17	0.18	0.18	0.19	0.19	0.20	0.21	Thionazin
0.19	0.20	0.20	0.21	0.21	0.21	0.21	0.22	0.22	0.23	0.23	Ethoprop
0.20	0.21	0.21	0.22	0.22	0.23	0.23	0.24	0.24	0.24	0.25	Phorate
0.21	0.22	0.22	0.23	0.23	0.23	0.24	0.24	0.25	0.25	0.26	Sulfotepp
0.23	0.23	0.24	0.24	0.24	0.25	0.25	0.25	0.26	0.26	0.27	Naled
0.23	0.24	0.24	0.25	0.25	0.25	0.26	0.26	0.27	0.27	0.28	Oxydemeton methyl
0.27	0.27	0.28	0.28	0.28	0.28	0.29	0.29	0.30	0.30	0.31	Diazinon
0.30	0.30	0.31	0.31	0.31	0.32	0.32	0.33	0.33	0.34	0.34	Diuxathion
0.32	0.32	0.33	0.33	0.33	0.34	0.34	0.35	0.35	0.35	0.36	Demeton thiol
0.30	0.31	0.31	0.32	0.33	0.33	0.34	0.34	0.35	0.35	0.36	Disulfoton
0.38	0.38	0.38	0.38	0.38	0.38	0.39	0.39	0.39	0.39	0.40	Diazoxon
0.40	0.40	0.41	0.41	0.41	0.42	0.42	0.43	0.43	0.44	0.44	Dichlorfenthion
0.46	0.46	0.46	0.47	0.47	0.47	0.48	0.48	0.48	0.49	0.49	Dimethoate
0.48	0.48	0.48	0.49	0.49	0.49	0.50	0.50	0.51	0.51	0.51	Ronnel
0.51	0.51	0.51	0.52	0.52	0.52	0.53	0.53	0.54	0.54	0.55	Cyanox
0.55	0.55	0.55	0.56	0.56	0.56	0.57	0.57	0.58	0.58	0.59	Ronnoxon
0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.61	0.61	0.61	0.61	Monocrotophos
0.58	0.59	0.59	0.59	0.59	0.60	0.60	0.60	0.61	0.61	0.62	Chlorpyrifos
0.57	0.58	0.58	0.59	0.59	0.59	0.60	0.60	0.61	0.61	0.61	Zytron
0.62	0.62	0.62	0.63	0.63	0.63	0.64	0.64	0.64	0.64	0.65	Fenthion
0.67	0.67	0.67	0.68	0.68	0.68	0.68	0.69	0.69	0.69	0.70	Malaoxon
0.72	0.72	0.73	0.73	0.73	0.73	0.74	0.74	0.75	0.75	0.76	Methyl parathion
0.81	0.81	0.80	0.80	0.80	0.80	0.79	0.79	0.79	0.79	0.78	Malathion
0.86	0.86	0.85	0.85	0.85	0.84	0.84	0.84	0.85	0.85	0.86	Fenitrothion
0.94	0.94	0.93	0.93	0.92	0.92	0.91	0.91	0.90	0.89	0.88	Bromophos
0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	Methyl paraoxon
0.93	0.93	0.93	0.92	0.92	0.92	0.91	0.91	0.91	0.90	0.90	Phenthoate
0.91	0.91	0.91	0.91	0.90	0.90	0.90	0.90	0.90	0.91	0.91	Bromophos ethyl
0.85	0.84	0.84	0.83	0.83	0.83	0.82	0.82	0.83	0.84	0.86	Schradan
0.96	0.96	0.96	0.96	0.97	0.97	0.97	0.97	0.97	0.98	0.98	Dicaphon
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	Ethyl parathion (REFERENCE)
1.02	1.02	1.02	1.01	1.01	1.01	1.00	1.00	1.00	1.01	1.01	Amidithion
1.02	1.02	1.02	1.02	1.02	1.03	1.03	1.03	1.03	1.03	1.03	Iodofenphos
1.11	1.11	1.10	1.10	1.10	1.10	1.10	1.10	1.09	1.09	1.09	Cruformate
1.18	1.18	1.17	1.17	1.16	1.16	1.15	1.15	1.15	1.14	1.13	DEF
1.19	1.18	1.18	1.17	1.17	1.16	1.16	1.15	1.15	1.14	1.13	Phosphamidon
1.18	1.17	1.17	1.17	1.16	1.16	1.16	1.15	1.15	1.14	1.13	Folex
1.25	1.24	1.24	1.23	1.23	1.22	1.21	1.21	1.20	1.19	1.18	Ethyl paraoxon
1.23	1.22	1.22	1.22	1.22	1.21	1.21	1.21	1.20	1.20	1.19	Methidathion
1.37	1.36	1.36	1.35	1.34	1.33	1.33	1.32	1.32	1.31	1.30	Tetrachlorvinphos
1.87	1.85	1.84	1.83	1.82	1.81	1.80	1.78	1.77	1.76	1.75	Ethion
1.89	1.88	1.87	1.86	1.85	1.84	1.83	1.82	1.80	1.79	1.78	Carbophenoxon
1.89	1.88	1.87	1.86	1.85	1.84	1.83	1.82	1.80	1.79	1.78	Carbophenothion
3.18	3.15	3.12	3.09	3.06	3.03	3.00	2.98	2.95	2.92	2.89	Phenkapton
3.96	3.92	3.87	3.83	3.79	3.75	3.70	3.66	3.62	3.58	3.53	Fensulfothion
4.65	4.60	4.55	4.50	4.45	4.40	4.35	4.31	4.26	4.21	4.16	Imidan
4.66	4.61	4.56	4.51	4.46	4.41	4.36	4.31	4.26	4.21	4.16	EPN
5.67	5.63	5.59	5.55	5.51	5.47	5.42	5.38	5.34	5.30	5.26	Famphur
7.48	7.38	7.27	7.17	7.06	6.96	6.85	6.75	6.64	6.54	6.43	Azinphosethyl
7.53	7.42	7.32	7.21	7.10	6.99	6.89	6.78	6.67	6.57	6.46	Azinphosmethyl
12.4	12.0	11.7	11.3	11.0	10.6	10.3	10.0	9.6	9.3	9.0	Coumaphos

is included to demonstrate this. Actual RRT determinations were made for 8 chlorinated compounds at 5° intervals, using a column of 1.5% OV-17/1.95% QF-1 on 100-120 mesh Chromosorb W (HP).

Proceeding on this information, the RRT values for all compounds were determined at 3 temperature points, 170, 185, and 200°C. Three injections of each of the multicomponent mixtures were made consecutively and the RRT_A or RRT_P values were calculated for each com-

pound. The 3 values per compound were averaged and the resulting value was used as the plotting point for construction of the slope curve. Aldrin was used as the reference compound for all electron capture chromatography (RRT_A), and ethyl parathion was used for flame photometric chromatography (RRT_P).

During each change of oven temperature, from 200 to 185°C, and from 185 to 170°C, the calibration procedure was repeated. During the course of each run at a given temperature, the

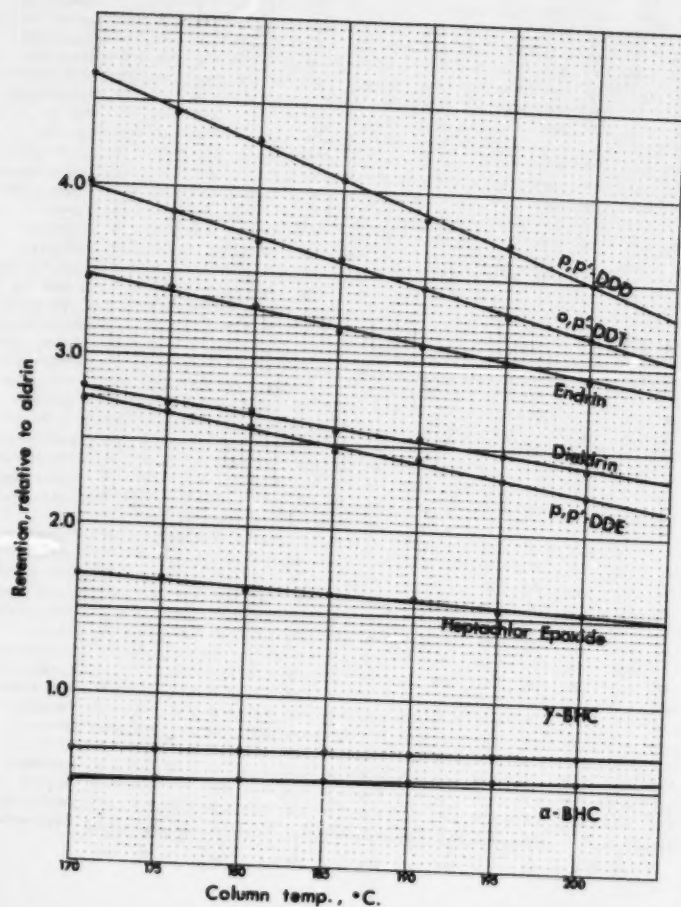


FIG. 1—Curves of 8 pesticidal compounds, plotting retention times, relative to aldrin, against column temperature at 5° intervals from 170 to 200°C. Column of 1.5% OV-17/1.95% QF-1 on 100-120 mesh Chromosorb W (HP).

oven temperature, as indicated by mercury thermometer and pyrometer, was constantly monitored.

Upon completion of all GLC work, a slope curve program was written for the computer. This program solved the equation of the regression line which provided the "best fit" to a set of x and y data points. The "normal equations" for a least squares line were solved simultaneously.

$$\Sigma y = bn + m\Sigma x \quad (1)$$

$$\Sigma xy = b\Sigma x + m\Sigma x^2 \quad (2)$$

obtaining

$$m = (n\Sigma xy - \Sigma x\Sigma y) / (n\Sigma x^2 - (\Sigma x)^2) \quad (3)$$

where n = the number of data points, each point consisting of a temperature (x) and a corresponding RRT (y).

Then the m value was utilized in solving equation 1, $b = (\Sigma y - m\Sigma x) / n$, where b = numerical distance on the x axis from the y axis and m = slope of a line.

At the top of each table, an arrow is placed over the temperature judged by the authors to be optimum for general usage. The criterion for these selections consisted of a compromise of the following factors: column efficiency, practicality of retention time in terms of work output, and resolution of the greatest number of pesticidal compounds.

Several compounds in the organophosphorus class exhibited multiple peaks via flame photometric detection on the 3 columns used, presumably resulting from the presence of impurities or decomposition products. These were naled, dioxathion, oxydemetonmethyl, azinphos-ethyl, carbophenoxon, Imidan, malaoxon, and fensulfothion. In each case the major peak was used for the RRT_P value.

The tables have proved very useful, particularly for tentative peak identifications in the chromatograms of multiresidue samples. First, it is necessary for the operator to establish the prevalent true column temperature. This is determined by chromatographing a standard mixture comprised of the appropriate reference compound (aldrin or parathion) and a relatively late eluting compound such as p,p' -DDT or Imidan. The RRT_A or P of the late eluting compound is calculated from the chromatogram. Then, by scanning horizontally across the col-

umn opposite p,p' -DDT (or Imidan) on the table, the RRT value is located which most closely matches the RRT value obtained by chromatography. The true column temperature can now be obtained by reference to the top or bottom of the table.

The sample extract is then chromatographed and the RRT_A or P values are calculated for all peaks appearing on the chromatogram. By vertically scanning the same temperature column on the table, the calculated RRT values may be compared with table values to obtain tentative peak identifications.

The system saves considerable time in that 2 chromatograms are generally sufficient to enable the operator to make tentative identifications of a number of the pesticidal compounds present in a sample. Using the more traditional approach, upon completion of the exploratory chromatogram on the sample extract, the operator usually selects reference standards on the basis of absolute retention values, or RRT values on the assumption that the column oven pyrometer readout is correct. If the actual oven temperature is significantly different from that indicated by the pyrometer, it may prove necessary to chromatograph a number of candidate reference standards before finding the compounds which produce comparable retention or RRT values.

Observations have occasionally been made that RRT_A values may shift slightly for certain organophosphorus compounds when they are chromatographed by electron capture detection on a given GLC column. Switching to an alternative column may result in an RRT_A value agreeing very closely with the table value. With this in mind, the chromatographer would be well advised to exercise caution in the identification of organophosphorus compounds determined by electron capture. Such compounds can be reliably identified by flame photometric detection as no such relative retention shifts have been observed in this detection mode when parathion has been used as the reference compound.

Conclusions

The data contained in the tables have proved very useful when coupled with the systematic approach described. The chromatographer is provided with a means of saving time and enhancing reliability in the tentative identification

of GLC peaks resulting from the injection of a substrate extract containing unknown levels of pesticide residues.

The analyst need not strive to precisely duplicate a specific column temperature given in a publication in order to match his relative retention values to those of the publication author(s). If one of the columns listed in Tables 1-9 is used, the analyst can determine his column temperature and then make tentative peak identifications from the RRT values given in the table.

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Measurement of Blood Cholinesterase

A Critical Account of Methods of Estimating Cholinesterase with Reference to Their Usefulness and Limitations Under Different Conditions

PUBLICATION

116

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Introduction

There is widespread interest in human red cell and plasma cholinesterases, and many methods for determination of these enzymes are available. However, a detailed evaluation of their comparative advantages has not been made recently.

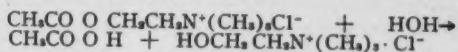
In this review, the principles upon which these methods are based will be discussed. An assessment will be made of the advantages and disadvantages of each procedure, and its expected accuracy will be indicated. In order to do this, a brief description of the properties of the red cell and plasma cholinesterases of man will be given. This description will be followed by a discussion of the general conditions required for the quantitative assay of these enzymes and a critical description of the principles of specific methods for their determination. Next, the problems common to all of the methods for the assay of blood cholinesterases and the difficulties which arise in the assay of blood samples from persons exposed to anticholinesterase insecticides will be discussed. Suggestions will be made in the text for future research to improve the ac-

curacy and reliability of the present methods and to develop new procedures that appear promising.

The viewpoint adopted will be that of the analyst. The question of interpretation of the results obtained with various procedures is outside the scope of this review except where the accuracy and reproducibility of the method is concerned.

Properties of Red Cell and Plasma Cholinesterase

In the body, 2 types of cholinesterase enzymes catalyze the hydrolysis of acetylcholine to acetic acid and choline.



Equation 1

The first type, acetyl or true cholinesterase, is of importance in the transmission of the nerve impulse (1) from preganglionic fibers to autonomic ganglia; (2) from postganglionic cholinergic nerves to smooth and cardiac muscle, and to secretory cells; (3) from motor nerves to striated muscle; and probably (4) from certain structures within the central nervous system. The physiological role of the second type, pseudocholinesterase, is unknown. True or acetylcholinesterase is found in the nervous tissue of all animals and in the red cells of most of them, and pseudocholinesterase is found in smaller amounts in the nervous system of all animals and in the blood serum of most of them.⁵ The cholinesterases can be differentiated from other nonspecific

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esterases, which also catalyze the hydrolysis of acetylcholine, by the sensitivity of the former to specific inhibitors such as eserine.¹¹⁹ True cholinesterase can be distinguished from the pseudocholinesterase by difference in substrate specificity between the 2 enzymes and by the use of inhibitors that are selective for one type of cholinesterase or the other.¹⁰ In general, maximal rates of hydrolysis of acetylcholine are obtained at lower concentrations of the choline ester with the former enzyme than with the latter. In fact, the true cholinesterase is inhibited by substrate concentrations that yield maximal rates of hydrolysis with pseudocholinesterase. However, the properties of these 2 enzymes differ greatly with the tissue of origin and the species under investigation.^{5,7} Therefore, the following discussion will be restricted to the properties of human red cell cholinesterase or human plasma cholinesterase.

Human red cell cholinesterase is a true or acetylcholinesterase; it is bound to the cell membrane²⁶ and is inhibited by an excess of acetylcholine.² The maximal rate of hydrolysis is obtained at 3×10^{-3} M solutions of this ester.² Although acetylcholine is the only known common, naturally occurring substrate of this enzyme, human erythrocyte cholinesterase will also hydrolyze the thion analogue of acetylcholine (acetylthiocholine),⁹³ propionyl choline,⁹⁶ acetyl- β -methylcholine,^{2,99} and acetyl- β -methylthiocholine.⁹

Human plasma cholinesterase is a pseudocholinesterase. Its optimal substrate concentration is about 2×10^{-2} M, and it is not inhibited by an excess of acetylcholine.² This enzyme catalyzes the hydrolysis of the thion analogue of acetylcholine⁴⁸ and the cleavage of propionyl¹¹⁷ and butyrylcholine¹²⁰ and their thion analogues.⁹³ The rate of hydrolysis catalyzed by the serum enzyme increases in the following order: acetylcholine < propionylcholine < butyrylcholine.⁶ As we shall discuss more fully later, the facts that the butyrylcholine and butyrylthiocholine are specific substrates for the pseudocholinesterase of human plasma, and that acetyl- β -methylcholine is a specific substrate for human red cells have been used as the bases for the

analysis of the activity of these enzymes in whole blood. The thion analogue of acetyl- β -methylcholine, acetyl- β -methylthiocholine, is not suitable for this purpose, because it is hydrolyzed by both serum and red cells.⁹ In addition to the substrates that have been used with whole blood for distinguishing red cell or plasma enzymes, plasma pseudocholinesterase also catalyzes the hydrolysis of a number of other esters both of choline and of other alcohols. Those of use in the determination of this enzyme are discussed in another section.

The properties of serum cholinesterase may be more complex than has just been suggested. Kalow et al. have obtained evidence from inhibitor and kinetic studies^{20,74-76} and by actual electrophoretic separation of the inhibited enzymes⁷³ that an atypical pseudocholinesterase may be found along with the usual cholinesterase in the sera of about 3.8% of a Canadian population of European descent. About 1 person in 2,820 has only the atypical cholinesterase. The condition was shown to be hereditary.⁷⁸ The atypical serum apparently does not have as great an affinity for choline esters as does the normal serum and, therefore, requires a higher concentration of acetylcholine to attain maximal rates of hydrolysis.

Other evidence indicates that "normal" sera may contain 2 or more cholinesterases. Heilbronn⁹² found evidence from electrophoretic and kinetic studies that 2 cholinesterases were present in a protein fraction from pooled plasma samples. Berry,¹⁵ from kinetic studies of the effects of substrate concentration, tentatively concluded that 3 cholinesterases are present in the sera of "normal" individuals.

Red cell and/or plasma cholinesterase are inhibited by a variety of compounds of which the quaternary ammonium bases,⁶ the phenothiazines,^{7,92} the organophosphorus compounds,⁹⁸ and the carbamates⁶ are the most powerful. The latter 2 are of particular interest because of their wide use as insecticides. The mechanism of the inhibition of cholinesterase by organophosphorus and carbamate compounds will be discussed later in

this review. Comprehensive lists of cholinesterase inhibitors have been compiled by Holmstedt^{6,8} and by Augustinsson.^{5,7,10}

Conditions Necessary for the Assay of Cholinesterase

Let us now turn to a description of the conditions needed for the assay of cholinesterase. All methods are based on a determination of the rate of hydrolysis of acetylcholine as given in Equation 1 or of a suitable synthetic ester that is catalyzed by the plasma or red cell cholinesterases. There are 2 prerequisites for a satisfactory procedure. The first of these is that the rate of the reaction measured must be proportional to the amount of enzyme present. In other words, a straight-line relationship must exist between enzyme concentration and enzyme activity. The second prerequisite—but of equal importance to the first—is that the enzyme measured under the conditions of the assay must be a cholinesterase. Usually this is demonstrated by showing that low concentrations of the specific inhibitor eserine inhibit the hydrolysis of the substrate. Differentiation of the type of cholinesterase requires the study of the rates of hydrolysis of various choline esters and the effects of other specific inhibitors. This subject is discussed in 2 excellent reviews by Augustinsson.^{7,10}

Several variables must be taken into account if conditions are to be found to meet the first prerequisite that the rate of the reaction must be proportional to the enzyme concentration. The rate of an enzyme-catalyzed reaction is dependent on the substrate concentration as well as the enzyme level but will become independent of the former if an excess of substrate is used. Therefore, in any cholinesterase method, the concentration of substrate should be such that its decrease during the assay procedure does not change the rate of the reaction. The fact that the optimal concentration of acetylcholine is lower for the red cell enzyme than for the plasma cholinesterase should be kept in mind. Also, the incubation period and the range of enzyme concentration to be tested should be adjusted

so that an excess of enzyme is not present. Thus, a straight-line relationship between rate of disappearance of substrate with time should be obtained. Procedures in which the amount of the substrate or product is measured at several time intervals, thus giving a curve that relates substrate disappearance to time, are preferable to those in which the rate is measured after one given period of time has elapsed. In the first method, corrections can be made for any deviations from a straight-line relationship. If the second method is used, it must be shown by other experiments that the rate of disappearance of substrate is linear with time within the range of enzyme concentrations used and over the period of time during which the rate is determined. Another method similar to the second procedure is to determine the time required for a certain percentage of the substrate to be used up.

The rate of an enzymatic reaction is also influenced by the pH of the medium. The optimal pH of human serum cholinesterase is in the range of 8.0 to 8.5,^{3,2} while the activity of this enzyme decreases at pH 11 and is destroyed at pH 2.0.^{4,12,8} The erythrocyte cholinesterase has an optimum pH at 7.5-8.0² but is less stable to alkali than the plasma enzyme and is denatured at pH 4.5.⁴ Therefore, in order to obtain a linear relationship between enzyme concentration and rate of the reaction, it is essential either to control the pH of the reaction mixture within narrow limits or, if this is not possible, to compensate in some way for the change in activity of the enzyme which occurs if the pH is allowed to change.

The activity of human red cell or plasma cholinesterase has been shown to be dependent on the presence of certain salts and on the ionic strength of the assay medium. The influence of ions on the enzyme activity is still a matter of controversy, but K^+ , Na^+ , Mg^{++} and Ca^{++} are considered activators, although the extent of the activation with the latter 2 ions is debatable.^{5,8,88} Satisfactory procedures have been devised for the determination of cholinesterase of plasma or red cells in which Ca^{++} or Mg^{++} is omit-

ted. Evidently enough Mg^{++} or Ca^{++} is present in these fluids to activate the enzyme. In these procedures an anticoagulant must be used, such as heparin, that does not combine with Ca^{++} or Mg^{++} as does oxalate or citrate. Potassium ion is considered an activator of red cell cholinesterase.^{5,88} Bicarbonate ion should not be used in the absence of chloride ion.¹¹⁸

Another important variable which must be controlled in the determination of the activity of cholinesterase is the temperature of the reaction mixture during the incubation period. For example, the rate of the hydrolysis of acetylcholine by serum cholinesterase increases 35% as the temperature is raised from 25 to 35 C,^{1,49,130} so a change of ± 1 degree at 25 C could cause a change of $\pm 4\%$ in the cholinesterase activity of serum. For results to be accurate, therefore, it is important to control the temperature at which the enzyme reaction is carried out. For some assays that are conducted at a moderate temperature, such as 25 C, a constant-temperature room free of drafts and regulated to ± 1 degree is satisfactory. In tropical areas, where it is difficult to maintain water baths or rooms at 25 C, it is advisable when setting up an assay to use a temperature of 30 C for the water bath, since water below this temperature can usually be obtained and can then be heated to the desired temperature. In field methods in which it is not possible to control the temperature, the effect of change in this parameter on the assay procedure should be known. The ambient temperature should be noted so that a correct interpretation can be made of the data obtained. Excellent examples of the effect of temperature on the results obtained in a field method are given by Davies and Nicholls²⁸ and Fleisher et al.³⁹

Specific Methods for the Determination of Cholinesterase

Most methods for the estimation of cholinesterase are based on the determination of the rate of disappearance of acetylcholine or the rate of formation of acetic or butyric acid from the hydrolysis of acetylcholine, acetyl- β -methylcholine, or butyrylcholine. The latter

2 are specific substrates for red cell and plasma cholinesterase, respectively. Because choline is difficult to determine, no methods are based on the estimation of this compound. Other procedures employ acetyl- or butyrylthiocholine, and the rate of formation of thiocholine or disappearance of the thio ester has been used to estimate both enzymes. Plasma cholinesterase can also be estimated by the hydrolysis of other substrates. Among those that have been used for the assay of this enzyme are benzoylcholine,⁷⁷ carbonaphthoxycholine,¹⁰⁷ procaine,⁷² β -naphthyl butyrate,⁵¹ and dicholine suberate.⁸⁴ The reviewer does not regard the use of noncholinesters as desirable except for special purposes, and the review will be restricted to methods in which choline esters are used.

Methods Based on the Measurement of Acid Production from Acetylcholine, Acetyl- β -Methylcholine, or Butyrylcholine.—Methods based on measurement of the acid production from acetylcholine, acetyl- β -methylcholine, or butyrylcholine include the titrimetric and manometric methods in which the actual amount of acid produced is measured and the electrometric or pH indicator methods in which acid produced is measured in terms of pH change in a buffer system.

Titrimetric Method: The principle of this procedure is to titrate the acid liberated during the hydrolysis of the choline ester with standard alkali; the pH is held constant, using either an indicator or a potentiometer. Certain problems are inherent in use of the indicator method. The continually changing color in the reaction mixture is often difficult to match with the color of the indicator standard. This difficulty may be due to the color of the blood or the plasma or to other factors such as the presence of activators or inhibitors that may influence the hue observed in the unknown. These difficulties are obviated if a potentiometer is used to measure the pH. If an automatic recording titratograph is used, a precision and accuracy comparable to that obtained with the Warburg manometric procedure can be obtained. Titrators are now available commercially.

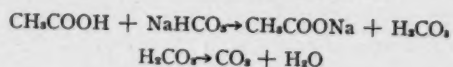
Procedures employing a recording potentiometric titration have been described by Chouteau et al.,²⁴ Jorgensen,⁷¹ and Jensen-Holm et al.⁷⁰ The time required for a titration by these methods varies from two⁷¹ to 10 minutes.²⁴ A straight-line relationship between enzyme concentration and enzyme activity is obtained.²⁴ The published procedures require a large volume of serum or blood (0.5 to 2.0 ml.) and large amounts of acetylcholine per assay. The procedures already mentioned were set up with blood serum with the exception of that described by Jensen-Holm et al.⁷⁰ which was also used with whole blood. At the Phoenix Field Station of this laboratory, microautomatic titration procedures based on the macro procedure and requiring 0.02 ml. of packed red cells and/or plasma have been set up and have been in use for 2 years⁴² with quite satisfactory results.

Certain precautions must be taken in order to obtain accurate results with the titrographs. Nitrogen should be blown over the solution in order to prevent errors due to the adsorption of carbon dioxide. To correct for any spontaneous acid production from the tissue, a preliminary titration should be carried out before the acetylcholine is added.⁷⁰ The ionic strength of the solution should be maintained at 0.1 to 0.2 and salts added as described in the first section. Also, the volume of alkali that is added should not be large enough to significantly increase the volume of the reaction mixture which would thereby decrease the enzyme concentration and change the rate of the reaction during the course of the assay.

Since little or no buffer need be present, the automatic titration method is applicable to the study of the effect of pH, substrate concentration, and other variables, such as salt concentration, on the activity of cholinesterase. Similarly, the method provides the best means to study the kinetics of the *initial* stages of the reaction of cholinesterase with inhibitors or reactivators. The Warburg method is not as useful in this application, because it requires 20 or 30 minutes to obtain a rate curve, whereas as little as 2 minutes are required with the titrimetric method.

The manual titrimetric procedures employing indicators are suitable for preliminary experiments to obtain an idea of the order of magnitude of an enzymatic reaction or to check results obtained with other procedures. They do not appear to offer any actual or potential advantages over the other methods discussed in this review. Augustinsson¹⁰ has prepared an excellent summary of these procedures, to which the reader is referred. Procedures have been described since Augustinsson's review in which the pH is allowed to fall for a standard length of time, and then the enzymatic reaction is stopped by the addition of eserine or similar inhibitor before titration begins. These procedures permit a delay in titration of the samples but have the disadvantages associated with a change in pH during assay as discussed below.^{53,60}

Manometric Method: The Warburg manometric technique is one of the most accurate, reliable, and versatile methods for the determination of cholinesterase; the precision and accuracy can be within $\pm 1\%$. In this procedure, the hydrolysis of the cholinester is carried out in a bicarbonate-carbonic acid buffer in an atmosphere of nitrogen-carbon dioxide in a closed vessel attached to a manometer. The acid liberated during the hydrolysis of the ester (Equation 1) reacts with the bicarbonate ion to form carbonic acid.



Equation 2

The carbonic acid concentration is fixed by the pressure of CO_2 in the gas atmosphere. Since the pressure is constant the carbonic acid disassociates and CO_2 is given off, thereby causing a change in gas volume which is measured at various time intervals with the manometer. Thus, a continuous record of the progressive hydrolysis of the ester is obtained, and an accurate rate curve can be plotted for each assay. Such a curve permits a more accurate calculation of the rate, and from the shape of the curve it is often possible to decide whether reactivation or inhibition has taken place during the assay.

A more detailed discussion of the theory and operating practice with the Warburg manometric procedure is available in the book by Umbreit et al.¹²⁹ Augustinsson¹⁰ gives an excellent description of the operating procedure for the determination of cholinesterase in red cells or plasma with the Warburg manometric procedure.

This procedure first was applied to the analysis of cholinesterase activity of human plasma and red cells by Ammon⁸ and has been used by numerous investigators since that time. Normal values have been published by Callaway et al.,²⁰ Ammon,⁹ and Augustinsson.⁸ The assay can be carried out with plasma, with separated red cells, or, as shown by Augustinsson and Heimbürger,¹¹ with whole blood using butyrylcholine or acetyl- β -methylcholine as specific substrates. In these procedures, a straight-line relationship between enzyme concentration and activity exists for the normal range of cholinesterase levels if 0.03 to 0.10 ml. of plasma or packed red cells are used.

However, this procedure cannot be used to study the effect of variation in pH on the reaction, since the practical range of pH is 7.2 to 7.7 with the bicarbonate-carbonic acid- CO_2 buffer. At higher pH levels the salt concentration may become too high. This method cannot be employed to study the rate of hydrolysis of cholinesterase at concentrations of acetylcholine below about 0.0004 M, because not enough gas is liberated to permit accurate measurement.

Sodium chloride should be present in the reaction mixture; since Smallman and Wolfe¹¹⁸ have found by comparison of the Warburg and titrimetric techniques that the absolute activity of cholinesterase is reduced by NaHCO_3 but that both methods give the same results when sodium chloride is also present along with the bicarbonate. It seems probable that 0.0015 to 0.04 M Mg^{++} or Ca^{++} should be present,¹⁰ but this is still somewhat a matter of controversy. The concentration of acetylcholine should be about 0.03-0.04 M for plasma and 0.003-0.004 M for red cells. It is necessary to make a correction for the nonenzymatic hydrolysis of

the acetylcholine when used at high concentrations. However, this rate is quite reproducible with a given batch of acetylcholine and can be subtracted from the observed rate to yield accurate and consistent results. Limitations in the use of the Warburg procedure in samples of blood obtained at autopsy are given in the last section of this review.

Other manometric apparatus that may be used in this assay offer no advantages over the Warburg method. Simplified flasks and manometers¹⁰ for the Warburg procedure are no more convenient to use than the standard Warburg apparatus which has the added advantage that it can be employed for manometric procedures other than cholinesterase determinations. Other manometric procedures have been reviewed by Augustinsson.¹⁰

Electrometric Method: This procedure is based on the determination of the rate of production of acid from acetylcholine or its analogs by instrumental measurement of the change in pH of a buffer. The pH of the buffer is measured with the glass electrode in a pH meter. The original method was developed by Michel,⁹⁴ and the procedure, which was carefully worked out, has been the basis of indicator as well as other electrometric methods.

The washed red cells are incubated in a phosphate barbiturate buffer at pH 8.0. The pH is noted at zero time and at the end of a definite period of time. The activity is calculated from the difference in the 2 pH values and is expressed as $\Delta \text{pH/hr}$ at 25 C. The acetylcholine concentration chosen for the red cell determination is at a slightly inhibitory level, but this concentration is necessary to permit the rate of disappearance of the substrate to be linear with time over the reaction period.^{20,94}

The plasma cholinesterase is determined in the same way, except that a higher acetylcholine concentration is used, and the buffer is more dilute to compensate for the inherently weaker activity of the plasma enzyme. The buffers have been designed so that the decrease in activity of the enzyme with pH over the range from 8.1 to 6.0 is compensated for

by a decrease in the buffer capacity; so there are only slight deviations from a straight-line relationship between decrease in pH and time during the incubation period. The buffers contain the activating salts needed for the 2 enzymes. Mg^{++} is not added to the buffer in order to avoid precipitation of phosphate; but potassium is added in its place, since this ion has some activating effect on red cell cholinesterase.^{5,88} Heparin should be used as an anticoagulant rather than oxalate or citrate, either of which may complex with the Mg^{++} or Ca^{++} present in the blood. At the concentration of red cells or plasma recommended by Michel, those tissues contribute only 12% and 7%, respectively, to the buffer capacity of the system, and larger amounts of tissue should not be used. Michel also determined corrections for the nonenzymatic hydrolysis of acetylcholine and for slight deviations of the ratio of change in buffer capacity to change in enzyme activity as the pH is lowered. The method has been found to give results parallel to those of the Warburg procedure.⁹⁴

A number of micromodifications have been devised.^{41,57,124,138} These are all essentially the same procedure as proposed by Michel except that a small sample of blood, usually 2 or 3 drops, is collected and separated into cells and plasma in a capillary tube. With the exception of the procedure described by Hamblin and Marchand,⁵⁷ the red cells are not washed. The results obtained for red cells with the micro procedure have been found to be about 5% to 7% higher than those obtained with the macro method.^{46,115,124} This difference is probably a reflection of the more efficient packing of the erythrocytes in the capillary tubes during centrifugation.

The Michel method is suitable for the analysis of large numbers of routine samples. Under optimal conditions, the precision obtained between duplicates with this method is ± 0.02 - 0.03 Δ pH units.^{45,79,100,138} The accuracy of the Michel and Warburg manometric procedures will be compared later. The most extensive single study of the cholinesterase values of normal persons was carried out by Rider and his colleagues¹⁰⁹; the re-

Cholinesterase Activity (Δ pH/hr) of Normal Human Blood *

	Men	Women
Red cells		
range	0.39-1.02	0.34-1.10
mean $\pm$ s.d.	0.766 $\pm$ 0.081	0.750 $\pm$ 0.082
Plasma		
range	0.44-1.63	0.24-1.54
mean $\pm$ s.d.	0.953 $\pm$ 0.187	0.817 $\pm$ 0.187

* The means (but not the ranges) are for people 40 years old. On the average, the plasma cholinesterase of men increases about 0.02 per decade and that of women increases about 0.04 per decade. The enzyme activity of red cells does not change with age.

sults are summarized in the Table. Other studies have shown that values exceeding the ranges in the Table are encountered in normal people but only very rarely.

One of the most common difficulties with the Michel method has been instability of the pH meter under the conditions of this test, in which pH is determined at intervals of one minute. The development of more stable pH meters in the last few years has greatly increased the usefulness of this procedure.

The pH of the buffer must not change more than 0.01 pH unit when acetylcholine is added. Most samples of acetylcholine chloride become unusable after a few weeks, even though stored in a desiccator. Acetylcholine perchlorate is more stable and, therefore, a more satisfactory salt to use. Change in the buffer capacity is another common source of error. This can be checked by titrating the buffer with 0.1 N hydrochloric acid; 10 ml. of the red cell buffer and plasma buffer should require 1.08 and 0.32 ml. of acid, respectively, to change the pH from 8.0 to 7.0.⁹⁵

Several electrometric procedures have been devised to assay for the plasma or red cell enzymes, using whole blood. MacDonald et al.⁸⁹ proposed a method in which a low concentration of acetylcholine is used. Under these conditions the major part of the activity measured was due to the erythrocyte enzyme as shown by the use of inhibitors. However, these tests were done with a blood with normal activity, and more interference from plasma enzyme might occur with samples of lower activity.

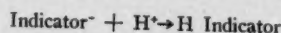
An electrometric procedure using acetyl- β -methylcholine as a specific substrate for red cell in whole blood⁸⁹ is more specific for the red cell cholinesterase. The accuracy and reproducibility of this microprocedure are the same as those of the Michel procedure. It was shown by the use of inhibitors and by the assay of plasma that this substrate was specific for red cell cholinesterase. Values for a normal population have been determined with this method.^{45,90}

Although the precision of the electrometric method based on the original procedure of Michel is high, the absolute accuracy or the sensitivity is not as great as those of the Warburg or titrimetric methods, because the acid production, itself, is not measured but rather the pH, which is a logarithmic function of the acid concentration, is determined. The pH is measured after one fixed time interval, which does not permit as accurate a determination of the rate as a procedure in which the activity is measured at several time intervals, since graphs relating change in pH to time are not usually constructed for each assay. Tammelin¹²⁶ has published a description of a recording pH meter which permits the simultaneous recording of curves relating drop in pH to time for 6 samples. In this procedure, the pH range for the determination is 8.0 to 7.7, instead of 8.1 to 6.0 as in the Michel method. Since the activity of cholinesterase is not affected over this narrow pH range, corrections due to changes in buffer capacity can be ignored. Tammelin and Strindberg¹²⁸ showed that with acetylcholine as a substrate and with separated red cells or plasma, the rate curves obtained were similar with the manometric and the modified electrometric methods when expressed in terms of μ M of acetic acid produced. The latter could be calculated in the electrometric method from calibration curves relating pH of solution to rate of addition of acetic acid.¹²⁷ Thus, the accuracy of the electrometric procedures is increased with the apparatus described by Tammelin et al. Cholinesterase levels of plasma and red cells in whole blood can be measured with this apparatus, using butyrylcholine or acetyl- β -methylcholine, re-

spectively.⁸¹ However, the latter substrate is split so slowly under the conditions used that the procedure is not a very sensitive test for the red cell cholinesterase.

Courville and Ledington²⁵ have introduced a modification of the electrometric method for serum cholinesterase which also increases its accuracy and precision. In this method, the activity is expressed as a function of the time required to change the pH of a bicarbonate-carbonate buffer from 8.2 to 7.8. It is claimed that the results deviate less than 1 second in 200, i.e., less than $\pm 0.5\%$. As far as the reviewer is aware, no application has been made of this more accurate procedure, although it would appear to be adaptable to automated analysis. However, this is not as elegant a procedure as that of Tammelin and Strindberg¹²⁸ in which rate curves are obtained with each assay.

Indicator pH Method: This procedure is based on the determination of the rate of production of acid from acetylcholine or its analogs by using an indicator to measure the change of pH in a buffer. The color change obtained is due to the formation of an acidic form of the indicator which has a different color than that of the basic form.



Equation 3

The ratio of these 2 forms, and hence the shade of color observed, is dependent on the pK of the indicator and the pH of the solution according to the well-known Henderson-Hasselbach equation.

$$\text{pH} = \text{pK} + \log \frac{\text{Indicator}}{\text{H Indicator}}$$

Equation 4

The change of the color of the indicator will approximate most closely a linear function of the decrease in pH if the pK of the buffer is no more than 0.1 pH unit from that of the indicator used.⁸⁵ Most investigators have neglected this factor.

A further complication is that indicators can combine with protein, particularly serum albumin, so that a change in color of the indicator, and hence an apparent decrease in pH,

can occur when, for example, phenol red-containing buffers are mixed with protein solutions such as serum.¹¹⁰ These errors are minimized, however, if dilute buffers and plasma solutions are used.¹²¹ If the indicator is present during the time the acetylcholine is being hydrolyzed by the enzyme, it must be shown that the indicator does not inhibit the enzyme. If the indicator is added at the end of the reaction period, the optical density must be determined without delay in order to obtain accurate results, because the enzymatic reaction does not stop with the addition of the indicator unless the latter is a potent inhibitor.

Most of the colorimetric procedures employing acid-base indicators have been devised for serum. In general, hemolytic, icteric, or lipemic sera cannot be analyzed. Only a few methods of this kind are available for the red cell enzyme. Semiquantitative field tests in which whole blood is used have been described. In this section no attempt will be made to cover all of the early literature. Examples of older methods and applications of new principles that have appeared since Augustinsson's review¹⁰ will be discussed.

Several procedures have been devised for the photometric estimation of serum cholinesterase. Most of these employ phenol red as the indicator. In the methods of Reinhold et al.¹⁰⁸ and Molander et al.,⁹⁸ the hydrolysis of acetylcholine is carried out in a phosphate-barbital buffer. After a standard incubation period for hydrolysis of the ester, phenol red is added and the optical densities at 540 m μ of a tube (A) containing no substrate and another tube (B) containing acetylcholine are determined. A calibration curve relating pH to the optical density of the phenol red solutions is prepared with a series of standard phosphate-barbital buffers of known pH containing phenol red and serum. From the optical densities of Tubes A and B, the initial and final pH, and hence the change in pH (Δ pH) is calculated.

A straight-line relationship between enzyme concentration is observed over most of the range of activity tested. The method is reproducible to ± 0.03 Δ pH; tables of normal values and the influence of pathological

conditions have been published.^{10,98,108} Slightly lower (0.03-0.04 Δ pH) results are obtained than with the Michel procedure. Greater deviation between the 2 methods is noted in sera of low activity.^{10,98,108} The error is probably of the order of 5%. Icteric, lipemic, or hemolyzed sera cannot be analyzed. In these procedures the concentration of phenol red must be rigidly controlled in order to minimize the error due to absorption of the indicator by serum albumin.

This difficulty is not encountered in the methods described by Caraway²¹ and Grégoire et al.⁵⁴ The procedure devised by Caraway is similar to those described in the previous section, except that the activity is calculated as a function of the ratio of the optical density of the tube with acetylcholine to the tube without substrate. Under these conditions, the phenol red concentration can vary $\pm 40\%$ with only a 2% change in the results. The enzyme activity is a linear function of serum concentration. The data are expressed in terms of the acetic acid liberated. This is determined from a calibration curve relating the amount of acetic acid present to the ratio of optical densities. The calibration curve is prepared from buffers of known pH containing known amounts of serum, phenol red, and acetic acid. Normal values for the serum enzyme determined in this way are given by Caraway.²¹

In the method of Grégoire et al.,⁵⁴ the phenol red is added to the enzyme solution containing Michel's barbital-phosphate buffer. The rate of the reaction is followed by measurement of the optical density at 535 m μ at various time intervals for a total of 8 minutes at 25 C. A graph relating optical density to time is plotted. The activity of the sample can be calculated from the slope of the line thus obtained and a calibration curve relating the slope of the line to known amounts of acetic acid added to the buffer plus serum and phenol red. Thus the enzymatic activity is obtained from a rate curve for each sample. The authors found a straight-line relationship between enzyme concentration and activity. The reproducibility of the method was about

1% to 2% for normal samples and about 5% for samples of low activity.

The procedures described by Caraway²¹ and Grégoire et al.⁵⁴ are an improvement over the older procedures using phenol red. In addition to minimizing the error due to absorption of phenol red by serum albumin, the interference due to CO₂ or acid fumes in the air is decreased, since stronger buffer solutions are used. The method described by the former author is suitable for the analysis of large numbers of samples; the procedure described by the latter authors requires a special attachment to maintain a constant temperature in the photometric cell compartment. A recording spectrophotometer would be of convenience for this procedure. Caraway²¹ has presented a modification that permits his procedure to be used with icteric or lipemic sera.

An automated analysis system employing the colorimetric determination of the pH change of a buffer with phenol red has been applied to the analysis of serum cholinesterase.¹³⁵ In this procedure, acetylcholine, barbitol-phosphate buffer, and serum are measured and mixed automatically and incubated at 37°C for a standard length of time. Then phenol red is added, the reaction mixture is dialyzed, and its optical density is measured in a photometer at 535 m μ and recorded on a recorder. No pipetting or handling of samples or reagents is required; the entire procedure is automated, and up to 40 tests per hour can be performed. A calibration curve is prepared with dilutions of purified cholinesterase. Excellent reproducibility of duplicate and triplicate samples is obtained. Because of the dialysis procedure, the error due to protein binding of phenol red should be at a minimum, although an excess of protein can prevent dialysis of an indicator. The dialysis procedure should also make it possible to assay lipemic or hemolyzed sera, although this was not tested. The method should be of great value in laboratories where large enough numbers of routine cholinesterase assays are done to justify the expense of buying the equipment and the time needed to learn to use it. Anyone wishing to use the

method will probably also want to prepare a calibration curve with buffers of known pH and with serum whose activity has been assayed by another procedure in order that the activity can be expressed as Δ pH or as μ M acetic acid liberated per unit time. The cholinesterase activities of a normal population have not been determined with this procedure. The method was not tested with human red cells, but there is no reason why a satisfactory procedure for erythrocyte cholinesterase could not be set up using this apparatus. The procedure has been used as the basis of a method for the determination of water-soluble organic phosphorus insecticide residues.¹³⁶

In one of the first published descriptions of a procedure for serum cholinesterase, *m*-nitrophenol was used as an indicator.²⁷ This indicator was reintroduced for the assay of serum cholinesterase by Rappaport.¹⁰⁶ This author points out that the absorption peak of this indicator does not change with variation in acidity and claims that the *m*-nitrophenol is not bound to serum albumin as much as is phenol red. The procedure and calculations are similar to those described by Caraway²¹ and, in the reviewer's opinion, the *m*-nitrophenol method offers no advantage. Icteric, hemolyzed, or turbid sera cannot be used. The normal values given are for children.

The indicator methods have not been applied as widely to the analysis of red cell cholinesterase as to the analysis of serum. This undoubtedly is because of the interfering color of the red cells. Bromothymol blue, which absorbs in the region of 620 m μ where the interference from hemoglobin is small, has been used for the quantitative estimation of red cell cholinesterase as well as plasma cholinesterase by Schiller¹¹³ and Biggs et al.¹⁶ In the method described by Biggs et al.,¹⁶ the assay is run on whole blood and plasma separately, and the activity of the red cells is obtained by difference. The method of calculation proposed by Caraway,²¹ i.e., of dividing the initial optical density of the solution by the final density, is used so that the dye concentration is not a critical variable. The concentration of acetylcholine that was chosen by Biggs et al.¹⁶ gives optimal rates

with the red cell enzyme but only 65% of the maximal rate with the plasma enzyme. The hematocrit must be determined in order to calculate the results with greatest accuracy. Normal values are given, but they are not comparable to those of other authors because of the low activity of the plasma enzyme. Although reproducible results are obtained with the methods described by Biggs et al.,¹⁶ a procedure employing isolated red cells would be preferable to the calculation of the activity of this enzyme by difference.

Field Tests for Cholinesterase: In the field a procedure is needed that will permit rapid screening of a large number of samples so that persons with significantly low cholinesterase levels may be found quickly. Such methods should be easy to perform and should require only simple equipment and comparatively few manipulations and reagents. For these reasons, indicator methods have been the basis of field tests.

Bromothymol blue has been used as an indicator in field tests for the cholinesterase activity of whole blood.^{28,33,34,39,83,104,112}

The earliest described field test is that of Limperos and Ranta,⁸³ in which the color of a solution of hemolyzed blood, acetylcholine, and bromothymol blue is noted after incubation under standard conditions. The relative activity of the blood is determined from the shade of color thus obtained. No color standards are used in individual tests. The authors describe the various shades of color that are given by blood of known activity. The relative activity of the given sample is determined from this information and the observed shade of color. A field kit with the necessary reagents and color comparison chart for this procedure is available commercially.

The test must be carried out at 37 C, and the authors suggest that the investigator can achieve this temperature by holding the reaction mixture against his body. However, it is difficult to control the temperature in this way, and it is also difficult to decide what may be the shade of color, without standards for comparison. Several modifications have

been suggested in an attempt to correct these difficulties. Edson and Fenwick⁸⁴ have described a procedure in which a series of 8 colored-glass standards are used to match the range of color produced in the method of Limperos and Ranta.⁸³

The activity measured with these standards matched that measured by the Michel method within limits of one color disk, that is, 12.5%. Correction for the effect of temperature is made in the following manner: Blood from a person with "normal" cholinesterase is assayed in a preliminary experiment at the prevailing ambient temperature. The time required for the reaction mixture to match the standard glass marked "100-percent activity" is noted. (Usually this time is 20 to 30 minutes; the time of incubation is decreased at higher temperatures and increased at lower temperatures.) Then the other samples are allowed to react with the acetylcholine in the presence of bromothymol blue for the time determined in the preliminary experiment, and the color standard matching the color of each sample is noted. It is obvious that the results for different groups of tests will not be strictly comparable, insofar as each group is based on a different "normal" standard. Therefore, results are not necessarily comparable from day to day. This does not necessarily detract from the value of the test for detecting marked depression of cholinesterase activity in individual workers.

A kit containing the reagents and the color comparator to carry out this test is commercially available.⁸³ Its successful use in the field during an episode of parathion poisoning among crop workers has been described.⁸⁰

Davies and Nicholls²⁸ have attempted in another way to determine the color of the reaction mixture and to correct for temperature variation in the procedure of Limperos and Ranta.⁸³ In their modification, the time required to reach a deep orange color corresponding to pH 6.7 is used as a measure of the cholinesterase activity. A standard for comparison is prepared from acetic acid, blood, and indicator; or a standard colored glass may be used. The effect of temperature can be calculated if the ambient temperature

is known because tables giving incubation times at various temperatures for normal, for 50%-inhibited, and for 25%-inhibited blood are given by the authors. The procedure of Davies and Nicholls was further studied and slightly modified by Fleisher et al.³⁹ Charts were constructed relating enzyme activity of whole blood to temperatures and giving the distribution of cholinesterase activity in a normal population. A phosphate buffer instead of an acetic acid solution is used to prepare the color standard. Methods for the determination of plasma or red cell cholinesterase in whole blood using the specific substrates butyrylcholine and acetyl- β -methylcholine also were devised by Fleisher et al.,³⁹ and charts were constructed relating enzyme activity of whole blood with these substrates to temperature and giving normal levels. The major drawback to the procedures using the specific substrates is the instability of commercially available salts of these compounds. Pokrovski¹⁰⁴ and Sándi¹¹² have described methods similar to those of Fleisher et al.³⁹

Herzfeld and Stumpf⁶³ have described a procedure in which an indicator paper prepared from acetylcholine and bromothymol blue is wet with plasma, and its color is compared at various time intervals with the color of standard papers prepared from indicator and buffers of known pH. A table relating time of incubation to per cent of normal activity and correcting for the effect of temperature is given. However, the highest temperature studied was only 27.5 C. The dry test papers are stable in a desiccator for several months. The authors were able to detect persons with low plasma cholinesterase activities due to pathological conditions, and a study of a normal population showed a distribution of activity similar to that obtained by the Warburg method used as a reference. As far as the reviewer is aware, no test has been made of this procedure under field conditions. The major practical difficulty peculiar to this procedure is the necessity for standard buffers which would be difficult to preserve under field conditions.

The field tests described previously are not as accurate as the laboratory tests. The error is probably of the order of 25%, although a smaller error of 12.5% has been claimed for the methods in which colored-glass standards are used.³⁴

The actual comparison of the color against standards is subject to more experimental difficulties under field conditions than in the laboratory. Uniform illumination is best assured by working indoors near a window with a sheet of white paper or cloth held at an angle behind the samples or comparator. If colored-glass standards in a color comparator are not used, the color of the sample can be best ascertained by use of a wooden block with slots cut in it in such a manner that light comes in from the back of the tube containing the colored solution. Such devices are described in textbooks on methods of clinical chemistry. For best results, the tubes used should be of uniform diameter.

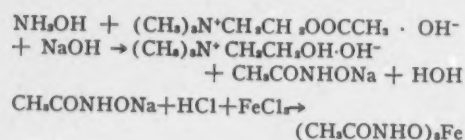
Other sources of experimental difficulty are the instability of the reagents. The indicator solution and the substrates must be adjusted to the desired initial color (initial pH) before use. Acetylcholine perchlorate is included in the field kit described above,³⁹ since it is more stable than the other salts of this ester.

The temperature at which the enzyme test is carried out greatly influences the enzyme activity observed. There are 2 possible methods for correction of this effect of temperature. They are: (1) use of a normal sample to adjust the time the enzymatic reaction is allowed to proceed, and (2) use of charts in which a temperature correction is given. The first method has the advantage that the standard normal sample is carried through all the steps of the test with the actual reagents used so that any variation due to differences in the observer's reagents and those of the original investigator are cancelled. On the other hand, the normal chosen may not have an "average" cholinesterase level, and misleading results could be obtained if one attempted the comparison of results of one group of tests with those of another. What is needed to improve the field methods are stable cholinesterase preparations of known activity which can be

used as reference standards so that absolute activities of the blood can be determined. Research along these lines would be most useful.

Methods Based on the Determination of the Rate of Disappearance of Acetylcholine.

The Hestrin Reaction: This procedure uses the reaction of acetylcholine in alkali with hydroxylamine to form acetohydroxamic acids which are then reacted in acid solution with ferric chloride to form a soluble reddish-purple complex. This color reaction first was adapted for the measurement of acetylcholine by Hestrin.⁶⁴ However, the reaction is not specific for acetylcholine but is given by other esters and anhydrides.³⁷



Equation 5

The method has been applied to the analysis of the cholinesterase activity of separated red cells,^{18,68,90,92,103} plasma,^{67,82,92,132} whole blood,³⁸ and red cells in whole blood.¹¹¹

A control tube containing no enzyme is incubated, along with an experimental tube containing enzyme, buffer, and acetylcholine. At the end of the incubation period, the color is developed. The amount of acetylcholine used up in the reaction is calculated by subtracting the optical density of the experimental tube from that of the control tube. The method is reproducible within $\pm 1\%$ and the accuracy is of the same order of magnitude. Only minute quantities, i.e., 0.2 μl to 50 μl of blood, are necessary for analysis with this method. The reaction mixture is stable for about 5 hours at room temperature after the addition of the alkaline hydroxylamine; but most authors agree that fading does occur after the addition of FeCl_3 ; hence, the optical density must be determined soon after the addition of the chromogenic reagent. A large excess of ferric chloride must be present; and since this

reagent has a slight absorbance at the wavelength (515 $m\mu$) at which the color complex has its maximal light absorbance, a blank, without acetylcholine, must be run with each determination and the color is read at 540 $m\mu$.⁶⁴

Benger and Kaiser¹³ found that the substitution of nitric acid for hydrochloric acid decreased the light absorbance of the chromogenic reagent at 515 $m\mu$ and thus reduced the blank. Under these conditions, the optical density of the standard or unknown could be determined at 515 $m\mu$. At the same time the rate of fading of the color was decreased. A method for blood cholinesterase in which nitric acid was substituted for hydrochloric acid has been published by Jensen-Holm et al.⁷⁰ It probably would be desirable for other investigators to determine whether the substitution of nitric acid for hydrochloric acid would improve the stability of the color complex under the conditions used in their laboratories. The conditions used by Pilz¹⁰³ also appear to stabilize the color.

Under the conditions of the tests cited in this section, a straight-line relationship between enzyme concentration and enzyme activity is obtained. Likewise the rate of disappearance of substrate is linear with time. The method of Fleisher and Pope³⁸ for whole blood actually measures mostly red cell enzyme, since at the concentration of acetylcholine used about 80% of the activity is due to that enzyme. The procedure described by Sabine¹¹¹ is a better measure of red cell cholinesterase in whole blood than the aforementioned method, since the plasma activity is inhibited with a specific inhibitor quinidine.¹²⁹ Red cell cholinesterase can also be determined on separated cells by the method of Metcalf⁹⁰; however, the plasma enzyme is more accurately determined by the procedure published by Huerga et al.,⁶⁷ since a low substrate concentration was employed by Metcalf⁹⁰ in his assay for this enzyme, and low results are thereby obtained.

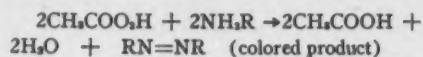
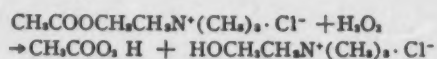
Since the amount of acetylcholine is determined by difference, at least 25% of the choline ester should be used up in order to obtain

the most accurate results. The Hestrin method for the determination of acetylcholine can be adapted to microprocedures employing 0.2 μ l of blood.¹⁸ It should be useful in routine investigations of cholinesterase levels and in studies of the effect of substrate concentration and salts. Bonting and Featherstone¹⁸ made a careful study of the effects of buffers and pH in the measurement of red cell cholinesterase and described conditions and buffers that permit the use of the Hestrin procedure to assay cholinesterase at various pH levels. The method is thus one of the most versatile and accurate procedures for estimation of cholinesterase activity, although rate curves are not obtained. Acetyl- β -methylcholine, butyrylcholine, and propionylcholine also react under the conditions of this test to yield colors of the same intensity, on a molar basis, as that produced by acetylcholine.¹⁸ Therefore, it should be possible to adapt this procedure by the use of specific substrates.

However, it should be kept in mind that a large excess of phosphate or borate ion can inhibit color development.⁶⁴ This is not a factor in the assays that have been discussed. Other carboxylic esters present in the solution to be assayed could yield interfering colors. Pilz¹⁰³ mentions that the lipids in lipemic sera might interfere, but in this reviewer's opinion the reaction with water-insoluble triglycerides would be so slow that color development from this source could be eliminated by using a shorter time interval between addition of the alkaline hydroxylamine and the acid ferric salt solution. A practical difficulty is the formation of gas bubbles after the development of the color which can cause difficulties in determination of the optical density. The reader is urged to consult the excellent papers by Hestrin,⁶⁴ Bonting and Featherstone,¹⁸ and Pilz¹⁰³ in which these variables are studied.

The Schönemann Reaction: Acetylcholine reacts with alkaline hydroperoxide to produce

a peracid which can then oxidize various amines such as *o*-dianisidine to produce a colored oxidation product.⁵⁸



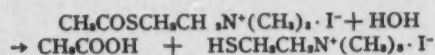
Equation 6

Unlike the Hestrin procedure, ordinary esters do not react. The color reaction is based on a reaction originally described for detection of organophosphorus war gases.¹¹⁴ This method has been applied to the analysis of organophosphorus insecticides⁴⁷ so that these substances might interfere in the analysis for acetylcholine. However, these phosphorus compounds also give the Hestrin reaction with alkaline hydroxamate. They would not be present in sufficient quantities to produce an interfering color if used at the concentrations ordinarily employed in enzyme studies.

The sensitivities of the Hestrin reaction and the Schönemann reaction as described by Hanker et al.⁵⁸ appear to be similar; that is, about 0.1 μ M to 0.4 μ M of acetylcholine per milliliter yield color densities in the useful range. However, it is possible that the sensitivity of the Schönemann reaction could be increased by the use of other amines whose oxidized products may have more intense light absorbancy or fluorescence.

Acetyl- β -methylcholine and succinylcholine also form colored products,⁵⁸ and other choline esters probably would react. The Schönemann reaction appears to be suitable for assay of cholinesterase by measurement of the rate of disappearance of acetylcholine in much the same manner as described for the Hestrin reaction in the previous section. However, the reaction has not been used except to assay the activity of a preparation of bovine erythrocyte cholinesterase.⁵⁸

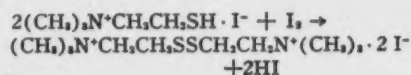
Methods Based on the Hydrolysis of Thiocholine Esters.—Several methods for the assay of cholinesterase are based on the hydrolysis of butyrylthiocholine or acetylthiocholine according to the equation:



Equation 7

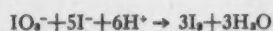
The rate of the reaction is measured: (1) by the rate of disappearance of the thioester band at 229 $\text{m}\mu$,⁴⁴ or (2) by the rate of the appearance of thiocholine.

The thiocholine produced has been determined iodometrically by Meyer and Wilbrandt⁹⁸ according to the equation:



Equation 8

and also by Augustinsson⁹ according to the equation:

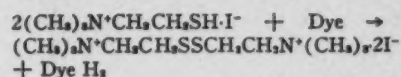


Equation 9

In the second procedure the iodine is generated by a more convenient method than in the first procedure.

A second method^{44,99} is based upon the decoloration of the blue dye 2,6-dichlorophenol-

indophenol by the thiocholine as in the equation:



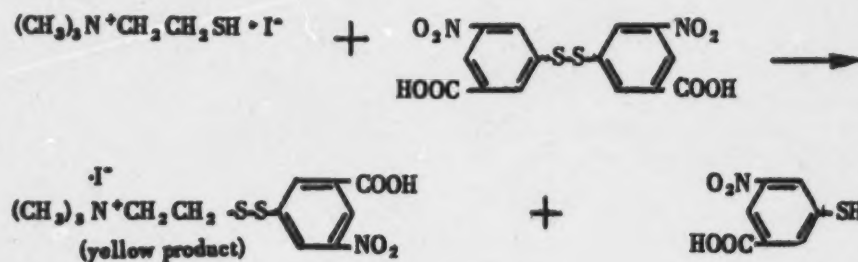
Equation 10

A third method³⁵ is based on the reaction of the thiocholine with dithiobisnitrobenzoate to yield a yellow color according to the Equation 11:

A fourth method¹²⁵ depends on the increase in absorption of the solution of 240 $\text{m}\mu$, which, according to Gal and Roth,⁴⁴ is caused by the formation of the iodide salt of thiocholines as a result of the hydrolysis of acetylthiocholine iodide according to Equation 7. This procedure has not been applied to any enzyme except purified plasma or erythrocyte preparations and is not as sensitive as other procedures.

A fifth method is based on the oxidation of thiocholine by sodium nitroprusside⁸⁷ to produce a yellow-orange reduced nitroprusside. The sensitivity of the reaction is of the same order of magnitude as the first 3 procedures. The color fades, and the optical density must be determined without delay after color development. This reaction has been applied to the estimation of the cholinesterases of rat tissues but not to human plasma or red cells.

Only one of the methods, that of Ellman et al.,³⁵ has been shown to be suitable for erythrocytes, even though the iodometric method (Equations 8 and 9) has been applied to the measurement of red cell cholinesterase in whole blood using acetylthiocholine as the



Equation 11

substrate in the presence of inhibitors of serum cholinesterase.^{9,33} However, a straight-line relationship between enzyme activity and enzyme concentration was not observed under these conditions by Augustinsson,⁹ who concluded that this method was not suitable for measurement of erythrocyte cholinesterase in whole blood. It is possible that a satisfactory iodometric method could be developed if separated cells were used.

In the procedure of Ellman et al.,³⁵ the red cell cholinesterase is measured in a small sample (10 μ l) of blood with acetylthiocholine at pH 8.0 as the substrate. The rate of the reaction is followed at 414 $m\mu$ in a spectrophotometer equipped with a recorder. The rate is calculated from the slope of the curve. It is necessary to run a blank without substrate to correct for turbidity of the cells and nonspecific sulphydryl groups in the red cells. The error of reproducibility is about 4%. The chromogenic reagent does not inhibit the enzyme nor does its rate of reduction limit the rate observed. Plasma cholinesterase is determined by difference, omitting the inhibitor. However, the activity of the plasma obtained in this way is not maximal, since the substrate concentration is too low for the pseudocholinesterase of plasma. There is a straight-line relationship between red cell enzyme activity and enzyme concentration over a 10-fold range; however, the data presented are for a purified erythrocyte cholinesterase of beef. Normal values have not been given.

Other procedures have been restricted, in the main, to plasma, purified bovine erythrocyte cholinesterase, and rat tissues. Spectrophotometric methods based on the disappearance of the 229 $m\mu$ band of the thioester⁴⁴ are suitable for the assay of this purified enzyme, and the indophenol dye method (Equation 10), in which the band at 600 $m\mu$ is followed, has been used for serum.⁴⁴ Ellman et al.³⁵ state that the light absorbancy of the yellow color produced during the reaction of thiocholine and dithiobis-nitrobenzoate is 2.5 times that of an equivalent amount of thioester measured at 229 $m\mu$, thereby increasing the sensitivity of the method. In addition, serum has a very in-

tense absorption in the region of 240 $m\mu$, and a shift of wave length to 235 $m\mu$ causes a twofold increase in light absorbancy.⁷⁷ Therefore, spectrophotometers of good resolving power must be utilized if the lower ultraviolet region of the spectrum is to be utilized. Iodide cannot be present if the 229 $m\mu$ band of the thioester is used to follow the reaction, because this halide ion has a very intense light absorbancy in this region of the spectrum. The commercially available iodide salt of acetylthiocholine must be converted to the less stable chloride.⁴⁴ Thus, procedures in which the near ultraviolet and visible portion of the spectra are used have several advantages over those in which the lower portion of the ultraviolet spectra is employed.

In all of these procedures, the auto-oxidation of the thiocholine is a factor to be considered. This factor is minimized in the spectrophotometric methods by the use of a fixed neutral pH of the reaction mixture, short reaction periods, and the use of de-ionized or glass-distilled water to decrease the contamination with copper which catalyzes the oxidation of the thiocholine or the dye. However, the rate of auto-oxidation of the latter 2 substances is markedly influenced by the pH. Although spectrophotometric methods employing thioesters provide sensitive procedures for the estimation of the cholinesterase activity of 0.001 to 0.01 ml. of blood, they are not as versatile as the titrimetric procedures and probably cannot be used for studies of the influence of pH on cholinesterase activity. As with the Warburg and the titrimetric procedures, rate curves are obtained with each spectrophotometric assay, thereby facilitating kinetic studies. However, in any kinetic experiments, the fact that the thioesters are not used at concentrations which provide a maximal rate of hydrolysis must be taken into account. These methods have not been applied to human red cells, to icteric or hemolyzed plasma, or in some cases to normal plasma.

The iodometric method (Equations 8 and 9) of Meyer and Wilbrandt³⁸ as modified by Augustinsson⁹ using butyrylthiocholine as the substrate for plasma cholinesterase in

whole blood does not give rate curves with each assay since the hydrolysis is stopped after a given length of time, and an iodometric titration (Equation 9) is carried out to estimate the thiocholine produced. This is a valid procedure, for a straight-line relationship exists between blood concentration and enzyme activity if barbiturate buffers are used,⁹ and butyrylthiocholine is not hydrolyzed by red cells.⁹³ As with other procedures, a blank containing no thioester must be run in order to correct for the reducing substances in blood that may react with iodine.⁹ The bromide salt of the thioester cannot be used, as bromide ion interferes in the iodometric titration. The accuracy of the method is about 5%. In the reviewer's opinion, this procedure appears to have no particular advantage over manual titrimetric methods. The iodometric methods might be of interest in laboratories in which a large number of these titrations were carried out for other purposes.

A proposed field test based on the reduction of the indophenol dye by whole blood, as in Equation 10, has not been tested except with a few normal samples in the presence and in the absence of diisopropyl fluorophosphonate.⁹³ It is possible that this observation might serve as the basis for a field test, since only a few reagents are required and the test is simple to perform. On the other hand, the procedure would have almost the same disadvantage as the field tests based on the use of acid-base indicators; that is, the color of the end point would be influenced by the color of the blood, and the rate of the reaction would be changed by temperature.

Methods Employing Other Choline Esters.

Methods employing choline esters other than those discussed in the previous sections

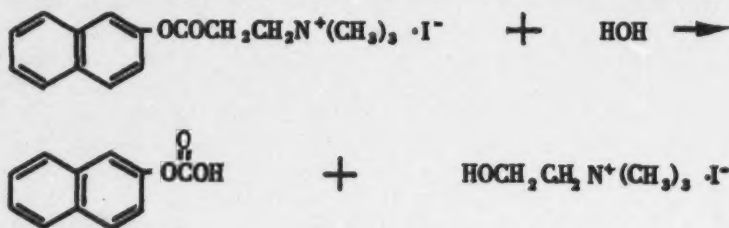
have been restricted to the measurement of plasma cholinesterase.

Carbonaphthoxycholine iodide is hydrolyzed by the serum enzyme to yield β -naphthyl carbonic acid according to Equation 12.

The latter decarboxylates spontaneously to yield β -naphthol. This alcohol is coupled with a diazonium salt to yield a dye that can be extracted with ethyl acetate and its optical density determined in a photometer. From 0.01 to 0.05 ml. of serum is required, and the rate of hydrolysis of the choline ester is proportional to the enzyme concentration over this range. If higher amounts of serum are to be tested, a correction for the absorption of the azo dye by plasma protein must be made. Normal values have been published for this method.¹⁰⁷ The accuracy of the method is probably of the order of 5%.

Benzoylcholine has been employed as a specific substrate for human serum cholinesterase using the Warburg manometric method. The pioneer studies of Mendel, Mundell, and Rudney⁸⁹ demonstrated that this ester was not hydrolyzed by enzymes present in erythrocytes. Kalow and Lindsay⁷⁷ have developed a spectrophotometric method for serum cholinesterase, employing 0.02 ml. of serum, in which the disappearance of the absorption band of the choline ester at 240 m μ is followed. Rate curves are obtained with each assay which can be carried out in 4 minutes.

A spectrophotometer with good resolution must be employed because of the marked change in the absorbancy of serum in the region of 230 to 240 m μ . The disadvantages of using the lower ultraviolet region have been discussed in the section on thioesters. Kalow and Lindsay⁷⁷ claim an accuracy greater than that of the manometric method.



Equation 12

The rates observed with the optical procedure are somewhat higher than those yielded by the manometric method. Many experiments employing benzoylcholine as a substrate have been done, and methods based on its use may be of particular value in the study of the kinetics of serum cholinesterase.

A method employing suberyldicholine for the assay of serum cholinesterase has been published. However, the paper is available to us in abstract form only, and it is not possible to make any further comment on it.⁸⁴

General Sources of Error in the Determination of Cholinesterase

Several sources of error in the cholinesterase assay are common to most procedures. First of all, the method of collecting heparinized blood must be standardized so that the sample is not contaminated with fumes of acid or alkali from the air or with alkali or anticholinesterase compounds from the skin of the subject who contributes the blood. The skin to be punctured must be washed free of these substances with ethanol which must be allowed to evaporate before the sample is taken.

Secondly, it is best, in general, to separate the plasma and red cells as soon as practical if analyses are to be run on the separate tissues. The cells should not be allowed to stand without a layer of isotonic saline over them. In the absence of saline, the cells have a tendency to clump, and they do not form an even suspension upon subsequent addition of isotonic solution.

Thirdly, the separated cells, plasma, or the whole blood should be kept at 0 to 5 C even during transport to the laboratory, because loss in enzyme activity can occur if the sample of blood, separated plasma, or red cells is kept at room temperature for more than a few hours.^{20,21,122} The claim that the blood enzymes are stable at room temperature for several days^{45,86} is not borne out by the experience of this reviewer or of the investigators cited above.

The separated plasma and red cell enzymes are stable for several weeks if kept at 0 to 5 C. The enzymes in whole blood are stable under these conditions, but the clotting of

the blood makes it difficult to work with. (The enzyme activity in the blood from men exposed to carbamate insecticides may not be stable as will be discussed below.) Plasma can be stored for several months in the frozen state. This is true of the red cell enzyme, also. However, unless the cells are hemolyzed before freezing, upon thawing, they become very difficult to pipette. Hobbiger⁸⁵ found that the cholinesterase of human red cells that had been frozen in solidified carbon dioxide (Dry Ice) is less stable to anaerobic incubation for 12 hours at 37 C than is the cholinesterase of fresh cells. Although these effects are less under aerobic conditions⁸⁶ and lower temperatures of incubation, they should be kept in mind when the enzyme of frozen cells is assayed.

Refrigeration of blood samples during shipment is an inconvenience. Some investigators have found that blood samples dried at room temperature on filter paper are more stable than liquid specimens. The dried samples can be kept at room temperature for 7 days and are stable for 3 months at 0 to 5 C when kept in a desiccator away from the acid or alkaline fumes.¹¹ Heilbronn⁸¹ states that the samples are stable for 5 months at room temperature. Blood can be spotted on the paper with a micropipet^{8,11} or allowed to drop on the paper to form a *uniform* spot.⁸¹ The dried papers then are wrapped and shipped to the laboratory without refrigeration.

In the laboratory, the sample measured with a micropipet can be used directly, and a known area is cut out of the spot made without pipets. The size of the aliquot is calculated from previous experiments or from the weight of the aliquot.⁸¹ Except for the most accurate results, the hematocrit need not be determined, since the variation in hematocrit is much less than the day-to-day fluctuations in cholinesterase levels.^{45,69,79} This procedure has been used successfully in the Scandinavian countries,^{8,40} and normal levels, as determined by the Warburg method using dried filter paper spots, have been published.⁸ This method of shipment of blood samples has not been tried in tropical coun-

tries as far as the reviewer is aware. To ascertain whether the stability of cholinesterase enzymes would be affected if blood samples were shipped in this manner under tropical conditions, dried samples of blood on filter paper should be subjected to testing at 30 to 40 C and high relative humidities.

Since the sample is whole blood, only methods that use specific substrates such as butyrylcholine or acetyl- β -methylcholine can be used to assay the plasma or red cell cholinesterase, respectively. Also, the red cell enzyme is poorly extracted, and about 80% remains bound to the paper.^{11,61} Therefore, the reaction of the substrate with this enzyme must occur in the presence of paper, and only those procedures such as the Warburg method¹¹ and the recording electrometric method⁶¹ in which the reaction mixture is agitated during the determination of activity can be used. Activities observed with this procedure are 3% lower for plasma and 10% lower for erythrocytes than the activities determined without the use of paper.⁸

Simmonds¹¹⁶ has proposed another way to preserve blood samples for shipment. The blood sample is applied to tablets of washed kieselguhr (0.05 ml. of blood to 24 mg. of kieselguhr) and allowed to dry at room temperature. The sample retains 85% to 90% of its activity for a week at room temperature and 80% for 2 months at 4 C. The pressed tablets retain their shape even with handling so they can be shipped. On the other hand, they are easily dispersed in the reaction media for analysis. The acetylcholine concentration employed in this study was such that the major part of the activity measured in the blood probably was due to the red cell enzyme. Since the enzyme levels were simply measured with shaking in the Warburg apparatus in solutions containing the powdered kieselguhr, no conclusion can be made from the data as to whether or not the erythrocyte or plasma enzymes, or both, were eluted from the absorbent. If these enzymes were readily eluted from the absorbent, it would be possible to use this method of preservation of the blood samples with pro-

cedures in which the reaction mixture is not shaken. Further research is needed to clear up these points.

Other variables that must be taken into account in assays of the cholinesterase of packed red cells are the conditions of centrifugation. For accurate laboratory measurements, the speed and duration of the centrifugation should be standardized. It is not safe to rely on rheostat readings for measurement of the speed of the centrifuge, since decreases in line voltage or defects in the motor can decrease the speed. Rather, it is best to check the speed of the rotor directly with a tachometer.

The cholinesterase enzymes of plasma and red cells are sensitive to heat. For example, the temperature at which the highest activity can be obtained has been found to be 37 to 40 C for the serum enzyme. Above this temperature, the inactivating effect of the rise in temperature on the enzyme begins to overcome the increased catalytic activity of the enzyme, and the observed activity begins to decrease and is completely gone at 70 C (for references see Augustinsson⁵). Therefore, it is essential to prevent overheating of the samples as might occur during centrifugation. If a refrigerated centrifuge is not available, the centrifuge tubes and their holders should be cooled before each centrifugation.

Another variable that is sometimes neglected in the analysis of separated red cells is the size of the centrifuge tube relative to the size of the sample of red cells to be quantitatively diluted with one volume of saline. For the usual 15 ml. conical centrifuge tube, 8 to 10 ml. of blood is the absolute minimum required for an accurate 1:1 dilution of the cells. Graduated centrifuge tubes of 2.5 ml. capacity are suitable for the accurate dilution of the cells from 1 to 2.5 ml. of blood. If undiluted, packed red cells can be prepared in a variety of centrifuge tubes; usually capillary tubes are used since this technique is employed, for the most part, with micro procedures.

Another source of difficulty in the assay of cholinesterase is the fact that the cholinesterases are inhibited by tertiary and

quaternary ammonium compounds.⁵ Many of the common detergents used for cleaning glassware are cationic compounds having the quaternary ammonium structure. One of the compounds commonly used as a disinfectant, cetyltrimethylammoniumbromide, is a potent inhibitor of cholinesterase.¹³³ Anionic detergents also can inhibit enzymes, so it is best to use nonionic detergents to clean glassware that is to be used for cholinesterase assays. If contamination with iron or copper ion is suspected, the glassware also should be cleaned with nitric acid.

Cholinesterase Assays on the Blood of Persons Exposed to Carbamate Insecticides

Insecticides of the carbamate type are coming into widespread use. These compounds have widely different inherent toxicities; the oral LD₅₀ for 4 commercially available ones ranges from 18 to 560 mg. per kilogram. Although inhibition of cholinesterase undoubtedly plays a major role in the toxic properties of the carbamates,⁹¹ the nature of the inhibition differs from that produced by the organic phosphorus compounds. This difference accounts for the greater difficulty of measuring the degree of inhibition produced by the carbamates.

Organophosphorus compounds phosphorylate cholinesterase to form a dialkylphosphorylated enzyme, but most carbamates (including the commercially available insecticides Dimetan, Isolan, Pyrolan, and Sevin) react to form only an inhibitor-enzyme complex which readily disassociates. It is true that the phosphorylated enzyme can be regenerated under certain circumstances, but this is a relatively slow process and the original inhibitor is destroyed.¹⁰⁰ Dialysis or dilution does little to promote regeneration of the phosphorylated enzyme.¹⁰⁰ On the contrary, cholinesterase can be rapidly regenerated from the carbamate-enzyme complex; the carbamate is unaltered in the process⁸⁰ and may be recovered by dialysis. (Certain of the carbamyl choline compounds are an exception to this rule; they may carbamylate

the active center of the enzyme in much the same manner as the organophosphorus compounds phosphorylate the enzyme.¹³⁴ However, the properties of these compounds will not be considered in this review, because they are not insecticides.)

The carbamate-enzyme complex is so easily disassociated that several factors discussed below have a marked effect on the equilibrium between enzyme and inhibitor. The ready reversibility of the inhibition of cholinesterase produced by carbamate insecticides renders measurement of the initial degree of inhibition very difficult. Enzyme activities by usual methods tend to be grossly too high.

These errors do not occur in the assay of this enzyme after inhibition with organophosphorus compounds, at least under ordinary circumstances. Kraye et al.⁸¹ and Burgen¹⁰ have described these errors associated with carbamates, and Goldstein et al., in a series of classical papers, have supplied a kinetic analysis of this problem.^{50,81,121} Myers⁹⁷ has extended the theoretical treatment of Goldstein et al. to a more general case. Kraye et al.⁸¹ list 3 sources of error in the analysis of cholinesterase activity in the presence of reversible inhibitors. These are (1) reversal of inhibition by dilution of the enzyme during preparation for the assay or during the assay itself; (2) reversal of inhibition by competition of the substrate with the inhibitor for the enzyme during the assay, and (3) destruction of the inhibitor before or during the assay.

The extent of the dilution error depends on the ratio of the concentration of the enzyme and the inhibitor. For example, Straus and Goldstein¹²¹ found that a 100-fold dilution of serum, which is the order of magnitude of dilution used in several of the commonly employed electrometric methods for cholinesterase, led to the following changes in the inhibition of the serum with the carbamate physostigmine. At a molar ratio of inhibitor to enzyme of 2:1, the calculated inhibition for undiluted serum was 43%; the observed inhibition of the diluted serum was 2%. At a molar ratio of 11:1, the calculated inhibition of the undiluted se-

rum was 96%, and the observed inhibition of the diluted serum was 20%. At a molar ratio of 215:1, the calculated inhibition of the undiluted serum was 100%, and the observed inhibition of the diluted serum was 76%. The dilution effect also varies with the disassociation constant of the inhibitor-enzyme complex, and the data quoted would apply to reversible inhibitors with an affinity for cholinesterase similar to physostigmine.

The reversal of inhibition by competition with the substrate is characteristic of reversible inhibitors and is revealed by a gradually increasing rate of hydrolysis of substrate during the assay. Excellent examples of the sort of rate curves obtained with physostigmine are given by Augustinsson and Nachmansohn¹²; the same sort of phenomenon was noted for 4 commercially available carbamate insecticides by Casida et al.²³ The condition of the assay employed by the former investigators eliminates dilution effects; the gradually increasing slopes in the rate curves given by the latter authors may include the effects of dilution as well as the effects of competition. A further complication of the competition of substrate and inhibitor for the enzyme is that the observed rate of inhibition is dependent on the substrate concentration; less inhibition is observed at high concentration than at low concentration of substrate. For example, 2×10^{-6} M prostigmine inhibits serum cholinesterase 10% at 3×10^{-2} M acetylcholine but 88% at 3.3×10^{-3} M choline ester.¹²

The third source of error mentioned by Kraye et al.⁸¹ is destruction of the inhibitor before or during the assay. The rate of hydrolysis of the carbamate insecticide by cholinesterase is very low. However, an enzyme that catalyzes the hydrolysis of *N*-methyl-substituted carbamates, such as the insecticide Sevin, is present in the albumin fraction of serum.²² Three other commercially available carbamate insecticides—Dimetan, Isolan, and Pyrolan—are of the *N*-dimethyl-substituted type, which is not attacked by this enzyme.²³ The action of this enzyme after the blood is drawn could reverse inhibition dur-

ing storage by mass-action effects and yield misleading high enzyme activities.

How can the analyst avoid or minimize these potential errors in the analysis of cholinesterase activities in blood containing carbamate insecticides? First of all, the blood should be chilled immediately upon drawing and analyzed as soon as practical in order to avoid enzymatic destruction of the inhibitor. Secondly, a method of assay should be chosen in which either specific substrates for red cell or plasma cholinesterase are used with whole blood or, if the plasma and erythrocytes are separated, packed red cells should be employed. Centrifugation should be in the cold, if possible. Thirdly, the dilution¹² and competition^{12,50} effects are not instantaneous but require from 10 to 25 minutes at room temperature to come to equilibrium, at least in the case of prostigmine, and the same delay probably is true of the carbamate insecticides. Therefore, methods should be chosen which permit measurement of the initial rate of the enzymatic reaction.

In order to accomplish this objective, it is necessary first of all to avoid dilution of the enzyme until it is added to the reaction mixture. Hemolysis of the erythrocytes should be accomplished with the lowest possible volume of saponin solution which should be made much more concentrated than in the usual assays. Secondly, initial rates of reaction can best be measured with methods of assay in which a short period of time is required to obtain a rate curve or in which the assay is carried out at a low temperature over a longer period of time. The recording titrimetric procedure in which rate curves can be obtained in 2 to 5 minutes would be suitable for the first procedure; the Warburg manometric method could be adapted readily for measurement at low temperature. The assay at low temperature is based on the assumption that the dilution and competition effects have the same or a greater temperature coefficient than does the catalysis of the hydrolysis of acetylcholine by cholinesterase.

Thirdly, the concentration of enzyme in the assay mixture should be as high and the concentration of acetylcholine should be as

low as is compatible with maintaining a straight-line relationship between the range of enzyme concentration studied and enzyme activity. The use of low temperatures of assay would permit higher concentrations of enzyme and, hence, tend to reduce the dilution effect. The concentration of acetylcholine employed should be reported with the results of the assay because, as pointed out previously, the inhibition observed is inversely related to substrate concentration.

Finally, the activity of a dialyzed sample should be compared to that of an undialyzed sample. Dialysis will remove the inhibition due to the carbamate compound. In this way an absolute normal for the individual just prior to the time of intoxication can be obtained and the per cent inhibition can be calculated without reference to other normal values, either of the individual himself or of the normal population.

As far as the reviewer is aware, no one has studied the inhibition of the blood cholinesterases of man or experimental animals exposed to carbamate insecticides by the methods just outlined. The kinetics of inhibition by these substances have been studied recently²³; but the data were obtained in experiments in which the dilution and competition effects were allowed to come to equilibrium so that maximal inhibitions would not have been observed. Research on the methods for the determination of cholinesterase activity of blood containing carbamate insecticides is needed.

Analysis of Autopsy Specimens

The analyst is occasionally asked to determine cholinesterase on specimens of blood obtained at autopsy in order to help determine whether death could have been due to poisoning with an anticholinesterase compound. Pribilla¹⁰⁵ compared the cholinesterase activity of sera from living persons and sera from the bodies of persons who had died from causes unrelated to poisoning. The results showed that the activity of this enzyme in serum was unchanged for a long time after death; determination was possible as long as unhemolyzed samples could be ob-

tained. Petty et al.¹⁰² found that the erythrocyte cholinesterase activities of 130 autopsy samples obtained 2 to 36 hours after death were within the range of normal values. Gage⁴⁸ also states that samples of blood taken at autopsy within 36 hours after death and then stored in the refrigerator will keep their activity for several months.

However, less is known about the stability of inhibited blood cholinesterases in the body after death due to poisoning with organophosphorus compounds. Dialkylphosphorylated cholinesterase formed from organophosphorus compounds containing dimethyl or diethyl phosphate groups can reactivate spontaneously but at a much slower rate than that of the enzyme inhibited with carbamates. The rate of spontaneous reactivation is a resultant of 2 opposing actions; (a) the spontaneous hydrolysis and subsequent reactivation of the dialkylphosphorylated enzyme (Type I), and (b) conversion of Type I to Type II which does not undergo spontaneous hydrolysis and is thus irreversibly inhibited. In vivo a third reaction, re-inhibition of spontaneously reactivated enzyme, occurs. Thus the activity of cholinesterase attained depends on the length of time the enzyme is in contact with the inhibitor (for review see O'Brien¹⁰⁰). It is theoretically possible that spontaneous regeneration of some cholinesterase activity could occur in the body after death or upon storage of samples if death occurred very rapidly upon ingestion of dimethyl or diethyl phosphorylated compound and if a large excess of the active form were *not* present in the blood at the time of death. On the other hand, the experiments of Bergner¹⁴ indicate that no reactivation of cholinesterase of muscle occurred in the carcasses of rats killed with lethal doses of reversible organophosphorus inhibitors. Using similar inhibitors in vitro, erythrocyte cholinesterase of animals was found to be reactivated spontaneously at 37 C if excess inhibitor was removed.^{17,31} However, studies of the stability of the blood cholinesterase in the carcasses of animals killed with a variety of compounds need to be car-

ried out in order to learn what happens when any one of the several variables changes.

It is generally agreed⁴⁸ that if the measured activity of a postmortem sample is less than 10% of the normal value, the evidence is strong that an organic phosphorus compound is the cause of death. Postmortem samples taken 4 hours after death from parathion poisoning have been found to average 10% of normal erythrocyte enzyme activity.^{55,56} On the other hand, the activity of this enzyme was 25% of normal in samples taken 13 hours after the death of a person from Systox poisoning.¹⁰¹ Also Pribilla¹⁰⁵ found the plasma cholinesterase of 11 parathion fatalities to be two-thirds or less of the normal activity. Thus it is clear that the finding of activity greater than 10% of normal in a postmortem sample does not rule out the possibility that death was caused by an organic phosphorus compound.

Proteolysis of the blood proteins can also cause a decrease in the level of cholinesterase activity by destruction of the enzyme. During proteolysis, acid is released; a simple test for this process is to compare the effect of the autopsy sample on the pH of a standard buffer with the effect obtained with a sample of fresh blood. Under the usual experimental conditions, 0.04 ml. of blood will not change the pH of 1 ml. of Michel's plasma buffer more than 0.02 to 0.04 pH units; the same volume of proteolyzed blood will decrease the pH 0.2 to 0.3 pH units instantaneously. If such a decrease in pH does occur, low cholinesterase values are equivocal and, in the absence of other information, no conclusion can be made regarding poisoning with anticholinesterase compounds from the results of such assays.

Extensively hemolyzed samples that are not proteolyzed can be assayed by the Nelson electrometric procedure or by the Warburg manometric procedure using specific substrates for the plasma or red cell enzymes. Whatever procedure is used, a test for proteolysis of the blood should be run.

Summary

The analyst has a variety of methods available for the assay of blood cholinester-

ase. The assay of serum or red cell cholinesterase can be carried out on the separated portions of blood or on whole blood using specific substrates. The most accurate and sensitive methods are the recording titrimetric and Warburg manometric procedures in which the amount of acid is measured stoichiometrically. Both of these methods are of great versatility and can be applied to the study of kinetics of inhibitors or activators and to the study of a variety of other experimental conditions, since rate curves are obtained. The Warburg procedure cannot be used for the study of the effect of pH beyond the range of 7.2-7.7; on the other hand, the titrimetric method can be used to study the entire useful pH range. The titrimetric method is of particular value in the study of the kinetics of the initial phase of the reaction between inhibitor, substrate or activator, and cholinesterase, since rate curves can be obtained within a period of 2 minutes. The titrimetric methods will become of increasing importance in this field.

The electrometric methods such as that of Michel, in which a decrease in pH is measured, are not as accurate as the titrimetric or manometric methods but are suitable for the assay of large numbers of routine samples. A modification of the Michel procedure using the specific substrate acetyl- β -methylcholine for red cell cholinesterase has been developed by Nelson. Rate curves are not obtained ordinarily with these procedures; but Tammelin et al. have described a recording potentiometric apparatus that permits the recording of rate curves relating change in pH to time of incubation, thereby improving the accuracy of the procedure.

Many procedures for serum cholinesterase have been described in which the decrease in pH of the buffer is followed by a change in color of an indicator, usually phenol red. An automated analysis procedure using this method has been described. The error due to absorption of the dye by serum albumin is reduced if the activity is calculated from the ratio of the final color to the initial color as proposed by Caraway or if the activity is calculated from the rate of decrease of the color

of the dye during the time the hydrolysis of the enzyme takes place. The accuracy of the latter 2 methods approaches that of the electrometric procedures.

Bromothymol blue has been used for the determination of red cell cholinesterase enzyme and for field methods in which whole blood is used. None of the field methods are entirely satisfactory, due largely to the lack of a suitable normal standard cholinesterase. However, useful results have been obtained with a field kit for a test based on matching the color of glass standards against the color of reaction mixtures after a necessary period of time for the reaction to occur. The other methods that are similar in principle are based on measurement of the time required for each reaction mixture to reach a certain color.

Methods for serum cholinesterase based on the measurement of the disappearance of thiocholine esters or the formation of thiocholine may have use in special studies of serum cholinesterase activity. The same is true of methods employing other choline esters, such as benzoylcholine, carbonaphoxycholine, and suberyldicholine.

The Hestrin method, in which the disappearance of acetylcholine is noted by means of the color reaction of this ester with hydroxylamine and ferric chloride, can, under proper conditions, have an accuracy which rivals that of the manometric or titrimetric methods. The method can be applied to a variety of experimental conditions and can be adapted to microprocedures. Since rate curves are not obtained during the determination of enzyme activity, this procedure is not as suitable for kinetic studies as the Warburg or the titrimetric procedures. However, the Hestrin method is useful for analysis of a large number of routine samples.

The Schönemann reaction, in which the disappearance of acetylcholine is noted by means of a color reaction of this ester with peroxide and a suitable amine compound, has not been applied to human blood. This color test is somewhat more specific for choline esters than the Hestrin reaction, and methods similar to those employing the Hestrin color reaction could be developed using the Schöne-

mann reaction for the detection of acetylcholine.

Because of the reversible nature of the inhibition of cholinesterase with carbamate insecticides, the usual methods for the analysis of the cholinesterase levels of blood give high results and thus suggest that the enzyme is less inhibited than it really is. Methods have been proposed to eliminate these errors and to obtain a closer approximation to the actual degree of enzyme inhibition in the plasma or red blood cells.

The analysis of autopsy specimens is discussed.

The errors involved in the determination of cholinesterase include possible contamination of the sample during collection, failure to centrifuge properly, and inactivation of the enzyme by keeping the blood too long at room temperature. Blood for analysis should be separated into cells and plasma and shipped under refrigeration. The stability of whole blood is increased by drying on filter paper, and blood prepared in this way can be shipped at room temperature. However, the red cell cholinesterase is bound to the paper. Further research is needed to ascertain the effect of tropical conditions on the cholinesterases of blood on paper. Blood cholinesterase can be preserved on kieselguhr; also, again, more research is needed to ascertain the effects on plasma or red cell cholinesterase and the stability under tropical conditions.

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A rapid and simple modification of the Dale, Curley and Cueto method for determining the prevalence of two organochlorine pesticides is found to be entirely satisfactory. **326**

PESTICIDES PROGRAM
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A SIMPLIFIED METHOD FOR ANALYSIS OF DDT AND DDE IN BLOOD FOR EPIDEMIOLOGIC PURPOSES

Small
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In an epidemiologic study to determine prevalence of the organochlorine pesticides p,p'-DDT and p,p'-DDE* in a representative sample of the population of south Florida, the problem presented to the laboratory was the need for a relatively rapid analysis of a large number of blood samples. Among possible methods offering a solution, several utilizing the gas chromatograph have been described.¹⁻³ The Dale, Curley and Cueto method was selected because modifications would further reduce the amount of time and number of manipulations required; yet, indications were that results would be comparable to the original method.

Essentially the modification described for the determination of DDT and DDE consisted of a standardized mechanical method of shaking, elimination of the concentration step described in the original method, and dilution of extracts which contained relative large amounts of the chemicals. Extraction with Nanograde™ hexane as solvent was carried out and the extract was injected directly onto polar and non-polar chromatographic columns. The resulting method was rapid, simple, reproducible, and suited to epidemiologic studies requiring large volumes of DDT and DDE data.^{4, 5, 6}

* All references are to the p,p' isomers. DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane. DDE: 1,1-dichloro-2,2(p-chlorophenyl) ethylene.

Results of a comparison of DDT and DDE values in whole blood and plasma determined by the modified method and the original method are reported, together with studies of reproducibility and percentage recovery of these residues from blood.

MATERIALS

Blood Specimens

Sixty-six whole blood specimens were collected; 15 from persons occupationally exposed to pesticides so that a wide spectrum of DDT and DDE concentrations would be provided. They were used for comparison of methods and of the whole blood and plasma values by the modified method. One also was utilized for the purpose of studying the reproducibility and percentage recovery of DDT and DDE after various shaking times, before and after spiking. Fifty of the specimens were analyzed so that effects of hemoconcentration might be studied by comparing DDT and DDE values in whole blood and in plasma.

Reagents

1. Hexane, Nanograde™, distilled in glass^a
2. Standard mixture of DDT and DDE prepared in concentrations of 20 ng/ml DDT and 10 ng/ml DDE^b
3. Ethyl acetate A.R.^a
4. Column packing: (a) Chromosorb

W-DMCS, high performance, 80/100 mesh and 100/120° (b) Chromport XXX, 60/70 mesh^a

5. Column Adsorbents: (a) QF-1, Dow-Corning FS-1265 fluid (fluorosilicone), viscosity 10,000 cs. at 25° C° (b) Dow-Corning silicone oil 200° (c) OV-1 methyl silicone oil°

^a Mallinckrodt Chemical Works, St. Louis, Mo.

^b Pesticides Research Laboratory, NCDC, PHS-DHEW, Perrine, Fla.

^c Applied Science Laboratories, State College, Pa.

^d Microtek, Inc., Baton Rouge, Louisiana.

NOTE: Commercial sources and trade names are provided for identification only. Their mention does not constitute endorsement by the U.S. Public Health Service or by the U.S. Department of Health, Education, and Welfare.

Equipment

1. Yankee Kahn shaker°
2. Gas chromatograph: Microtek 220 with electron capture detector (Ni⁶³) in DC mode. Glass columns: 6 ft. by 1/4 in. diameter. Columns: 5 percent QF-1 on Chromosorb W

Operating parameters: Inlet 220° C; column 185° C; detector 250° C; transfer line 240° C; voltage 35-40; input nitrogen pressure 40 psig.; N₂ flow rate 90 cc/min for QF-1 and 100 cc/min for Dow-200 and OV-1.

3. Centrifuge°
4. 10 ml glass stoppered graduated centrifuge tubes
5. B-D Vacutainers heparinized[†]
6. Micro-Hematocrit°

[°] Clay-Adams, Inc., New York.

[†] Becton, Dickinson and Co., Rutherford, N.J.

METHODS AND PROCEDURES

Blood samples (10 ml) were collected by venipuncture in heparinized B-D

Vacutainers and stored in the laboratory at 4° C until analyzed.

This modification of the Dale, Curley and Cueto method was performed: One milliliter of whole blood was extracted with an equal volume of Nanograde™ hexane in a 10 ml centrifuge tube. The extract was placed in a Yankee Kahn shaker for 20 minutes and then centrifuged for 2 minutes at 2,000 rpm. An aliquot of the hexane extract (5A) was injected onto the chromatographic columns using the maximum practical sensitivity of the detector.

Dilutions of the extract were prepared when large amounts of the pesticides were encountered so that peaks would appear within the linearity of the detector. The extracts were not concentrated in contrast to the original method.

Analyses were completed within the working day to minimize errors due to evaporation. At least two columns were used, and occasionally a third, to facilitate identification of peaks.

The Dale, Curley and Cueto method was then performed as published.¹

Utilizing the described method, reproducibility and percentage recovery of DDT and DDE from blood at various shaking times were studied in the following manner: 12 extracts were prepared from one blood sample. Six aliquots were analyzed in three pairs, each pair consisting of one unspiked aliquot and the other spiked with 20 ng/ml of DDT. Each pair was shaken a different length of time, i.e., 20 minutes, 1 hour and 2 hours. The other six aliquots were analyzed in three pairs, one of each pair unspiked and the other spiked with 10 ng/ml of DDE. Each pair was shaken for the same three different lengths of time.

RESULTS

A comparison of DDT values in ppb in the 15 whole blood specimens obtained by the Dale, Curley and Cueto

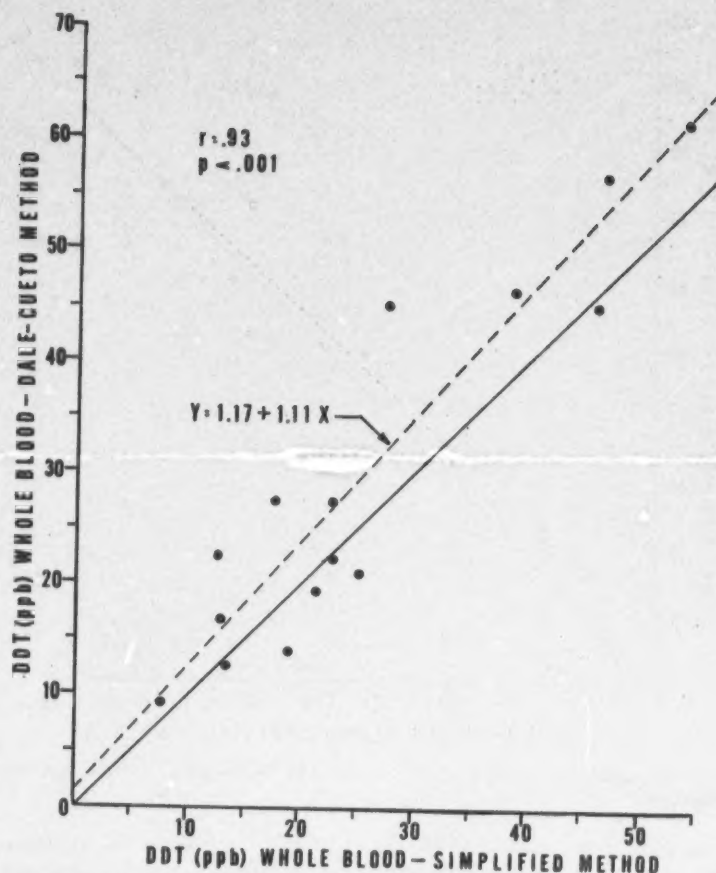


Figure 1—Comparison of DDT (ppb) Values, by Dale-Cueto Method and this Simplified Method.

method and by the modification described is presented in Figure 1. These specimens, obtained from a group of men occupationally exposed to pesticides, assured a wide range of values. The correlation coefficient (r) obtained in this comparison of DDT levels was (r)=0.93, $p < 0.001$. The slope is indicated on the figure.

A comparison of DDE values in ppb obtained in the same specimens by the two methods is presented in Figure 2. The correlation coefficient was (r)=0.92, $p < 0.001$. The slope is similarly indicated in Figure 2.

Results are presented in Table 1 of a study of reproducibility of the modified method and percentage recovery of DDT and DDE from spiked samples as compared to unspiked samples at various shaking times, that is, 20 minutes, 1 hour and 2 hours. The study was performed upon one collection of blood from one person. Reproducibility was obtained on repeated testing of the same blood for DDT (2 ppb repeated six times) and DDE (9 ppb repeated six times) which was not altered by lengthening the shaking time beyond 20 minutes. On spiking the specimen with

DDT-DDE DETERMINATIONS

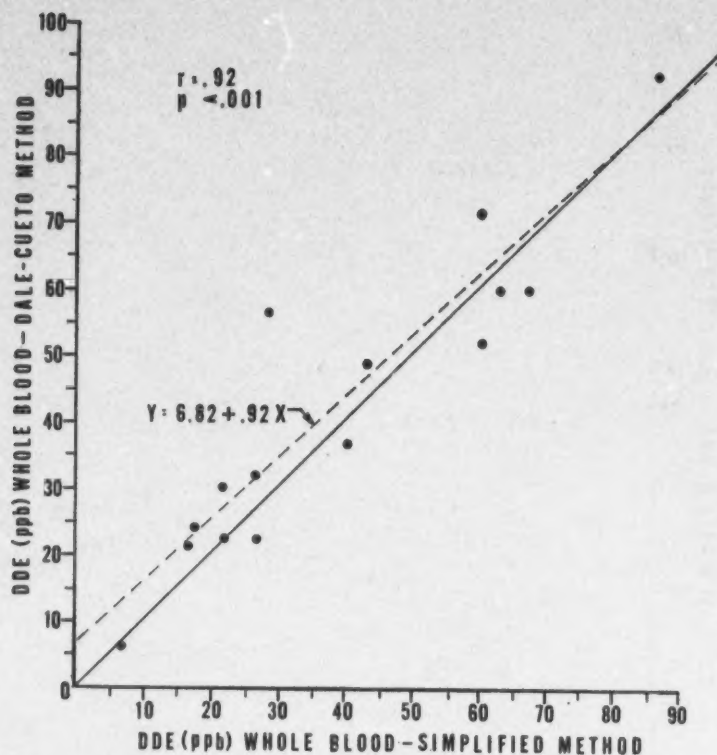


Figure 2—Comparison of DDE (ppb) Values, by Dale-Cueto Method and this Simplified Method.

20 ng/ml of DDT, recovery of DDT was over 90 percent regardless of shaking time; upon spiking with 10 ng/ml of DDE, recovery of DDE was 100 percent to 110 percent at the various times. Twenty minutes then was considered an adequate shaking time to obtain satisfactory recovery.

Fifty blood specimens were analyzed as whole blood extracts and as plasma extracts by the described modification; in 44 (88 percent) plasma values for DDT and DDE were comparable, within 3 ppb, to twice the whole blood values (50 percent hematocrit assumed). Of the six specimens in which DDT and DDE were not comparable within 3 ppb, all hematocrit determinations were above 40 percent. No common characteristic

appeared to account for the variance in these values. In the 50 specimens, however, considering plasma values as twice the whole blood values, the correlation coefficients (r) for DDT and DDE in plasma and blood were 0.91 and 0.91 respectively, $p < 0.001$. When plasma values were corrected to the actual hematocrit, the correlation coefficients (r) for DDT and DDE in plasma and whole blood values also were (r) = 0.91, 0.91, $p < 0.001$.

Identification and measurement of pesticides and their metabolites other than DDT and DDE were often encountered in these analyses, but are not included in this discussion since the epidemiologic studies^{4, 5, 6} were only concerned with the most ubiquitous and

Table 1.—Shaking Time, Reproducibility and Percent Recovery of DDT and DDE in ppb from Whole Blood.

A. Spiking with DDT					
Sample	Shaking Time	Reproducibility	DDT added	DDT found	% Recovery
1	20 min.	<2	None	<2	N/A
2	20 min.	<2	20	20	>90
3	1 hour	<2	None	<2	N/A
4	1 hour	<2	20	21	>95
5	2 hours	<2	None	<2	N/A
6	2 hours	<2	20	20	>90
B. Spiking with DDE					
Sample	Shaking Time	Reproducibility	DDE added	DDE found	% Recovery
7	20 min.	9	None	9	N/A
8	20 min.	9	10	21	110
9	1 hour	9	None	9	N/A
10	1 hour	9	10	19	100
11	2 hours	9	None	9	N/A
12	2 hours	9	10	19	100

predominant pesticide DDT, and its metabolite DDE.

DISCUSSION

Results obtained from analysis of 15 whole blood samples by the described modification of the Dale, Curley and Cueto gas chromatographic method for identification and measurement of chlorinated hydrocarbon pesticides in whole blood compared favorably with the original method. The correlation coefficient (r) was 0.93, 0.92 $p < 0.001$ for DDT and DDE respectively. In addition to being adequately comparable, the simplified method conserves laboratory time and proved satisfactory for analysis of a large number of blood specimens in epidemiologic investigations of the prevalence of DDT and DDE.

In a study designed to test reproducibility of the described modification and the percentage recovery of DDT and DDE, analysis of the same blood 12 times verified reproducibility for both residues. Recovery in spiked blood samples was over 90 percent for DDT and 100 percent to 110 percent for DDE. This was considered adequate for pur-

poses for which the test was designed. Comparison of shaking times revealed that 20 minutes was sufficient in both instances. Longer periods did not increase the percentage of recovery or alter reproducibility of the method.

Other methods have been described for measurement of DDT and DDE residues in whole blood, plasma, or serum and since, at times, plasma or serum may be more available than whole blood, a comparison of whole blood and plasma values obtained by the described modification was performed. Forty-four (88 percent) of the 50 specimens analyzed for DDT and DDE in plasma and whole blood compared within 3 ppb, when plasma values were halved. Six specimens did not similarly compare. It appears that DDT and DDE in some specimens are not predictably partitioned between red blood cells and plasma. For practical purposes, however, a hematocrit of 50 percent can be assumed and plasma or serum values can be considered twice those of whole blood. In extreme degrees of hemoconcentration or hemodilution, we suspect that this empirical conversion frequently will be inaccurate.

Sensitivity of the electron capture detectors varies from day to day, especially for DDT. At times DDT is measurable in blood or serum at 2 ppb. DDE is regularly measurable at the 1 ppb level. Since whole blood specimens from the general population often contain DDT in less than measurable amounts, it would be advisable to analyze plasma or serum rather than whole blood if lower levels than these (2-7 ppb) are considered important.

It is our practice, whenever possible, to measure DDT and DDE on the chromatogram from the QF-1 column especially if the residues are in a very low range. However, confirmation by other columns always is carried out. For epidemiological surveys the simplified method described appears suitable for identification and measurement of DDT and DDE, especially since DDT is an evanescent residue highly dependent upon recent exposure.⁶ In occupationally exposed individuals, levels of DDT less than 7 ppb are of little interest and are highly variable day to day.

SUMMARY

A modification of the Dale, Curley, and Cueto method for the determination of p,p'-DDT and p,p'-DDE in whole blood is described. It offers the advantages of rapidity and simplicity over the original method in the laboratory analysis of a large number of blood samples. Reproducibility and percentage recovery of the organochlorine pesticide residues were found entirely satisfactory. The modification was comparable, (r) =

0.92, to the original method when used with whole blood. Plasma and whole blood values of DDT and DDE were found to be unrelated to actual hematocrit corrections when readings were between 40 percent and 54 percent. It is speculated that this would not be true with blood specimens exhibiting high degrees of hemoconcentration or hemodilution. The correlation of both DDT and DDE concentrations in plasma with those in whole blood was (r) = 0.91 when the hematocrit was assumed to be 50 percent.

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ANALYTIC METHODS FOR PESTICIDES AND
ANTIBIOTICS IN FOOD*

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Modern antibiotics and pesticides have given man many advantages in his fight to control the bacterial and vector-borne diseases. Public health also has obtained many indirect benefits through improved agricultural production made possible by better pest control. The specific benefits from the use of antibiotics and pesticides are too well-known to this audience to require enumeration.

The wide-spread use of any biologically active chemical, and particularly its dissemination in food, involves a potential hazard, which must be evaluated. By necessity, first estimates of the safety of a pesticide must be based on its toxicity to animals. The evaluation of chemicals that may occur in trace quantities in food is based on animal tests and the results of residue measurements made possible by analytical methods.

Analytical methods for determining pesticides have been reviewed in some detail by Gunther and Blinn (1955), Metcalf (1957), and Westlake (1959). The following discussion will briefly review selected methods for determining the more common pesticides and will indicate some of the compounds which, for some reason, require particular attention in residue analyses of food.

Any discussion of methods of analysis for pesticides probably should begin with the most widely applicable method, which is bioassay. In bioassay techniques, various investigators have used a wide range of animal species, including primarily insects (houseflies, mosquito larvae, or *Drosophila*) and fish. Harwood *et al.* (1960) recently have reported an interesting technique in which they utilize differences in toxicity of various pesticides to four different organisms — including the pomace fly, the rusty grain beetle, a stored-products mite, and the brine shrimp — to qualitatively identify as well as quantitatively determine the pesticides. The bioassay technique is of great value in screening foods for the presence of pesticides generally and also in determining insecticides, particularly recently-introduced compounds for which no specific methods are available. Special cases of the bioassay procedure involve measurement of toxicants by their effects on isolated organ systems or on enzymes. Organ systems, although widely used in experimental pharmacology, have found little application in the pesticide field. The enzyme technique is extensively used in analysis for the anticholinesterase insecticides and will be discussed further below.

Paper chromatography is a useful tool in pesticide analysis, particularly in instances in which mixtures of pesticides are encountered. Gas chromatography coupled with thermal conductivity, flame ionization,

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or coulometric determination is a more recently introduced analytical method. It has been applied successfully to the chlorinated hydrocarbon materials and to the organic thiophosphates. In its present state of development, however, gas chromatography is not a routine type procedure; and its use should probably be limited to laboratories in which a person specifically trained in this technique is available to carry out the analyses.

Other generally useful analytical methods include determination of ultraviolet and infrared spectra. Since the absorption spectrum of a given compound is specific, it provides a means of both qualitative and quantitative analysis.

Specific colorimetric methods are available for most of the common pesticides and are usually the procedures of choice in residue analysis. A number of these methods will be discussed below under specific compounds.

The more widely used of the modern synthetic insecticides are either chlorinated hydrocarbon or organic phosphorus compounds. Although there is considerable overlapping in toxicity between these groups, on the average, the acute toxicity of the organic phosphorus groups is somewhat greater than that of the chlorinated hydrocarbon compounds. However, the chlorinated hydrocarbon compounds, due to their greater stability, are considerably more of a residue problem than are the organic phosphorus materials.

The chlorinated hydrocarbons are lipid-soluble and tend to accumulate in body fat in animals exposed to them. Lactating animals that have been exposed also excrete the chlorinated hydrocarbon materials in milk. Since milk is the primary food for infants and invalids, who might be unusually susceptible to the effects of toxicants, the U. S. Food and Drug Administration has not granted a tolerance level higher than zero for residues of any pesticide in milk. Recently, there has been much activity related to testing for various chlorinated hydrocarbon pesticides, especially DDT, in milk. In a 1958 survey conducted by the U. S. Food and Drug Administration, Clifford *et al.* (1959) reported that 23 (2.5 percent) of 936 samples of raw milk analyzed showed chlorinated hydrocarbon residues of 0.1 ppm or more. DDT was the offender in 15 of these samples. The other eight contained BHC, toxaphene, chlordane, or DDD. These authors felt that there had been a definite improvement in the milk residue picture since an earlier survey was made in 1955 (Clifford, 1957).

Some of the common chlorinated hydrocarbon pesticides are listed in table 1, along with their oral LD_{50} values to white rats and the tolerance level allowed by the Food and Drug Administration in certain items of food. In connection with studies of residue in foods, particular attention should be given to three of these compounds. DDT is still the most widely-used of the newer synthetic pesticides. Any program for the routine testing of food materials for pesticide residues almost certainly would include DDT. As yet, no definite tolerance has been granted for endrin on any foodstuff, due perhaps to the high tox-

icity and long residual properties of this compound. Heptachlor at one time had a tolerance of 0.1 ppm on a number of food items. However, additional work indicated that on plants, as well as within the animal body, heptachlor was changed to heptachlor epoxide; and this epoxide appeared, in some tests, to be more toxic than the parent compound. Therefore, a zero tolerance level has been placed on heptachlor, pending further clarification of this conversion process and of the toxic hazard of heptachlor epoxide.

The determination of total organic chlorine is widely used for the estimation of chlorinated hydrocarbon pesticide residues. This method involves decomposition of the organic molecule using a combustion furnace, sodium peroxide and heat in a Parr bomb, or metallic sodium in refluxing isopropyl alcohol (Gunther and Blinn, 1955) to liberate inorganic chloride ions. Then, the chloride may be determined by titration with standard silver nitrate solution. Concentration of the pesticide may be calculated by multiplying the chloride concentration by the proper factor.

Specific colorimetric methods are available for most of the more common compounds in this group, including all those which were listed in Table 1.

Aldrin, dieldrin, and endrin can be determined by the phenyl azide method, (O'Donnell *et al.*, 1954). For aldrin, phenyl azide is added in *n*-hexane; the solvent is removed by evaporation; and the residue is heated to form an aldrin dihydrophenyltriazole. Excess phenyl azide is removed by distillation under vacuum. The triazole is dissolved in ethyl alcohol and coupled with diazotized 2,4-dinitroaniline in the presence of hydrochloric acid. Sulfuric acid is added, and the optical density is determined at 515 $m\mu$. The major difficulties in this procedure are associated with the phenyl azide. If this reagent is not freshly-prepared, large reagent blanks are encountered. It is also necessary that the excess phenyl azide be completely removed before proceeding with further steps in the analysis. Caution should be exercised when handling phenyl azide, since this material is explosive.

Recently, Cueto (1960) has published an alternative procedure for quantitative determination of dieldrin. This technique involves the purple-colored complex formed by the reaction between dieldrin and diphenylamine in the presence of zinc chloride. The method is general for most chlorinated hydrocarbon insecticides and was applied earlier by Graupner (1960) for determination of toxaphene. However, the test is made essentially specific for dieldrin by use of saponification followed by chromatographing through an alumina column. The interfering materials are held on the column, while dieldrin may be eluted by an ether-hexane mixture.

Chlordane may be estimated by the specific colorimetric method of Davidow (1950). This method depends upon the formation of a red color when chlordane, diethanolamine, and potassium hydroxide are heated together in methyl alcohol. Rotenone and heptachlor interfere in this reaction, since they also give a red color under the conditions described.

The Schechter-Haller (1945) colorimetric procedure is the method of choice for DDT. This method involves nitration of the DDT, separation of the tetranitro derivative, and production of a chromophore from this derivative by treatment with alcoholic sodium methylate. Under these conditions, DDT forms a blue color (maximum absorption at 596 $M\mu$), while its metabolite, DDE, yields a red color (maximum absorption at 515 $M\mu$). Both dichlorodiphenyl acetic acid (DDA) and kelthane (hydroxy-DDT) form red colors in the above reaction. They can, however, be separated by chromatography.

The colorimetric reaction described by Polen and Silverman (1952) is favored for measuring residues of heptachlor. Heptachlor in benzene or hexane solution is treated with ethanolamine and potassium hydroxide in butyl cellulose to form a pink to violet color. Chlordane interferes in this analysis.

Gunther and Blinn (1957) have developed an ultraviolet spectrophotometric method for kelthane, utilizing its absorptivity at 264 μ . These authors also proposed an indirect method for kelthane determination, based on the absorptivity of its 2,4-dinitrophenyl-hydrazone in alcoholic alkali at 510 μ .

Lindane is determined colorimetrically by use of the Schechter-Hornstein method (1952). This technique involves dechlorination to benzene by means of zinc and acetic acid. The benzene formed is nitrated, primarily to *m*-dinitrobenzene that is treated with methyl ethyl ketone in the presence of strong alkali to produce a violet-red color.

The acaricide Tediion is one of the newer compounds in this group. It has just come into use on a commercial scale this year. Nitrated Tediion produces a red color with an absorption maximum at 520 μ when treated with alkali in the presence of pyridine (Fullmer and Cassil, 1958).

Some of the commonly encountered organic phosphorus pesticides are listed in table 2, along with their LD_{50} values and tolerance levels. The determination of total organic phosphorus is generally applicable to the estimation of these compounds. However, due to its non-specificity, to the high blanks encountered, and to its relatively low sensitivity, this method is not very useful in the determination of residues. The most generally helpful group-wise reaction for the organophosphorus insecticides involves *in vitro* cholinesterase inhibition (Giang and Hall, 1951). In this procedure, an aliquot of the extract to be analyzed is incubated with standard cholinesterase. The degree to which the enzyme is inhibited gives, on comparison with a standard curve, a measure of the amount of pesticide present. The method is made more sensitive by oxidation or other pre-treatment of the less active compounds to convert them to more potent cholinesterase inhibitors.

There are specific colorimetric methods available for many of the organic phosphorus pesticides. Since this group of compounds is not of great significance from a residue standpoint, analyses for only two of them, malathion and parathion, will be discussed here. Malathion

is determined by a procedure described by Norris, Vail, and Averell (1954). The procedure involves decomposition by alkali to dimethyl fumarate and sodium, o,o-dimethyl dlthiophosphate. The latter compound is converted to its cupric salt, which possesses an intense yellow color in carbon tetrachloride solution. The method of choice for parathion determination is the Averell-Norris technique (1948). This procedure involves reduction of the parathion and diazotization and coupling of the resultant amino compound with N-1-naphthyl-ethylene-diamine to form a purple color.

A few miscellaneous pesticides, their LD₅₀ values, and tolerance levels are listed in table 3. Aminotriazole has been found to be carcinogenic in studies with experimental animals and has a zero tolerance level. Aramite has produced hepatocarcinomas in rats and dogs on prolonged oral dosing and now also has a zero tolerance level.

Green and Feinstein (1957) have developed a method for determining aminotriazole, consisting of diazotization, followed by coupling with chromotropic acid. The absorbance of the resulting colored complex is read at 525 M μ . Brokke *et al.* (1958) have published a colorimetric procedure for aramite residues, based on hydrolysis and oxidation to formaldehyde. The formaldehyde is reacted with phenylhydrazine and sulfuric acid to yield a brilliant red compound with an absorbance maximum at 520 M μ .

Sevin (1-naphthyl N-methyl-carbamate) may be determined by reaction with p-nitrobenzenefluoroborate to form an intense blue color (Miskus *et al.*, 1959). This method permits independent determination of Sevin and of any 1-naphthol that may be present as a breakdown product. Although it is not an organic phosphorus compound, Sevin is a cholinesterase inhibitor and also may be measured by the method of *in vitro* cholinesterase inhibition (Moorefield and Tefft, 1958).

In studies on pesticide residues in food, in addition to studying single compounds, attention also may be directed toward combined residues. Regulations of the FDA state that, where two or more pesticides with similar pharmacologic actions are present, the combined percentile residues shall not exceed one hundred. Thus, the usage of two or more of these related chemicals on a single crop raises the possibility of exceeding the allowable tolerance for the combination of these materials.

The major problem associated with antibiotics in food materials involves residues in milk. The antibiotics, and especially penicillin, may get into milk when they are used in treatment of cattle diseases by intramuscular injection or intramammary infusion, when they are added directly to milk as a preservative, or when cattle are fed crops that have been treated with antibiotics to control plant diseases. Penicillin has very strong sensitizing properties and even the very small amounts occasionally found in milk might cause reactions in highly sensitive individuals (Jester *et al.*, 1959). Zimmerman (1959) has reported four cases of chronic urticaria from the United States and Vickers *et al.* (1958) have described two cases of acute dermatitis which were at-

tributed to penicillin in milk or other dairy products. The available tests for penicillin in milk, all of which are bioassays, include disc assay (Arret and Kirshbaum, 1959), cylinder plate method (Stoltz and Hankinson, 1953), microscopic tests (Liska, 1959), and reduction tests (Neal and Calbert, 1955).

In earlier surveys carried out by the U. S. Food and Drug Administration, 3.2 percent of the milk samples tested in 1954 were found to contain penicillin. In 1955, 11.6 percent and in 1956, 5.9 percent of the samples tested were contaminated. In the latest reported survey, which was carried out in 1959, 3.7 percent of samples contained penicillin and 2.0 percent contained other antibiotics.

SUMMARY

The wide-spread use of biologically active chemicals, including pesticides and antibiotics, involves a potential health hazard. Measurement of residues of these materials in foods is a necessary part of the evaluation of this hazard. Although the organic phosphorus insecticides are, in general, more toxic, the chlorinated hydrocarbon compounds present more of a residue hazard due to their greater stability. The major problem associated with antibiotics in food materials involves residues in milk. Analytical methods applicable to some of the common pesticides and antibiotics are reviewed.

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Table 1. Acute Oral LD₅₀ Values to White Rats and Tolerance Levels in Food for Chlorinated Hydrocarbon Pesticides.

Compound	LD ₅₀ Value (mg./kg.)	Tolerance Level (ppm)
Aldrin	39	0.25
Chlordan	335	0.3
DDT	113	7.0
Dieldrin	46	0.25
Endrin	18	Zero
Heptachlor	100	Zero
Kelthane	1,100	5.0
Lindane	88	10.0
Tedion	14,700	2 (citrus), 5
Toxaphene	90	7.0

Table 2. Acute Oral LD₅₀ Values to White Rats and Tolerance Levels in Food for Organic Phosphorus Pesticides.

Compound	LD ₅₀ Value (mg./kg.)	Tolerance Level (ppm)
Delnav	43	2.08 (citrus)
Demeton	6.2	0.75
Diazinon	108	0.75
EPN	36	3.0
Ethion	179	1.0
Guthion	13	2.0
Malathion	1375	8.0
Parathion	13	1.0
Phosdrin	6.1	1.0
Schradan	9.1	Zero
Trithion	30	0.8

Table 3. Acute Oral LD₅₀ Values to White Rats and Tolerance Levels in Food for Miscellaneous Pesticides

Compound	LD ₅₀ Value (mg./kg.)	Tolerance Level (ppm)
Aminotriazole	14,700	Zero
Aramite	3,900	Zero
Lead Arsenate	1,050	1 (citrus), 7
Sevin	850	10

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GAS CHROMATOGRAPHIC ANALYSIS OF PENTACHLOROPHENOL IN HUMAN
URINE BY FORMATION OF ALKYL ETHERS

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PENTACHLOROPHENOL (PCP) is an herbicide, defoliant, and antimicrobial chemical used throughout the United States as a preservative agent for many products. Pentachlorophenol seems to be present everywhere, appearing in municipal water supplies, wells, paints, wood and paper products, and in urine of every person now being examined (1). The ubiquity of human exposure to this potentially dangerous compound has prompted concern in the field of public health (1-6). This interest has been stimulated by several recent industrial (3, 4) and public (5, 6) intoxications which resulted in fatalities.

This report describes a simple and rapid method for the determination of PCP based upon its conversion to any one or more of seven possible alkyl ethers. PCP ethers are detected and quantitated by electron capture gas chromatography and their identity is confirmed by p -values. Data are presented for seven GLC columns and three p -value solvent systems.

Materials and MethodsApparatus:

A Micro-Tek MT-220 gas chromatograph, with tritium electron capture detector, was used. Inlet temperature was 220°C, column temperature 180°C, and nitrogen flow 80 ml/min. Gas chromatographic columns were U-shaped glass, 6' x 1/4". Glassware included a Kuderna-Danish evaporative-concentrator (Kontes K-569000); 15 and 1 ml test tubes; 15 and 5 ml ground glass-stoppered test tubes; 5, 0.4, and 0.1 ml pipettes.

Reagents:

Sodium hydroxide and hydrochloric acid, both analytical reagents. N-Methyl-N'-nitro-N-nitrosoguanidine, N-ethyl-N'-nitro-N-nitrosoguanidine, N'-nitro-N-nitroso-N-propylguanidine, N-isobutyl-N'-nitro-N-nitrosoguanidine, N-n-butyl-N'-nitro-N-nitrosoguanidine, N-isoamyl-N'-nitro-N-nitrosoguanidine, N-amyl-N'-nitro-N-nitrosoguanidine (Aldrich). Hexane, methanol, acetonitrile, dimethylformamide (Mallinckrodt, Nanograde).

Partition and Extraction:

Five ml of urine is transferred to a 15 ml ground glass-stoppered centrifuge tube. The urine sample is made alkaline by addition of 0.3 ml of 0.4 N NaOH. Five ml of hexane is added to and mixed with the alkaline urine sample. The layers are separated by centrifugation and the hexane layer is discarded. The alkaline urine is then made acidic by addition of 0.2 ml of concentrated HCl. The acidic urine is extracted by thorough mixing with exactly 2 ml of hexane. The sample is centrifuged and an aliquot of the hexane layer is then ready for derivatization.

Diazoalkane Generation:

(Use extreme caution; see Discussion.) Five ml of 20% NaOH is added to a 15 ml test tube. A 3 ml volume of hexane is placed on the 20% NaOH solution. One hundred mg of the nitrosoguanidine reagent is slowly added to the hexane in 10 mg increments (use a hood with a good draft). The reagent is ready for use after bubbles of the excess diazoalkane gas are no longer observed being evolved from the hexane solution.

Alkylation of PCP:

An aliquot, 0.4 ml, of the hexane extract of the acidic urine is pipetted into a 5 ml ground glass-stoppered test tube and then 0.1 ml of the alkylating reagent in hexane is added. The solution of diazoalkane plus hexane extract is mixed and allowed to stand. After 20 min, or a longer interval if

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convenient, 1 ml of 20% water in methanol is added to the reaction mixture and the two phases are mixed thoroughly. After phase separation, the hexane layer is ready for gas chromatographic and *p*-value examinations.

Gas Chromatographic Determination:

An aliquot, preferably 5 μ l, of the alkylated PCP in hexane is injected into a gas chromatograph equipped with an electron capture detector and a suitable column (see Table I). Peaks are compared to peaks produced by injecting the same volume of standard PCP-ethers. Larger volumes (up to 20 μ l) may be injected if similar volumes of diluted standards are used for quantitation. For peaks that are off scale, a smaller aliquot is taken or the sample is diluted.

If it is necessary to concentrate the sample, the organic solvent solution of PCP or PCP-ether is pipetted into an evaporative-concentrator tube. A two-bubble micro Snyder column is added and the tube is heated with steam, avoiding bumping and splashing, until the desired volume is obtained (7).

TABLE I

Retention Times of PCP-Ethers Relative to Aldrin^a

PCP Ether	10% Apie- zon L 80-100 Gas Chrom Q	3% DEGS, 80-100 Gas Chrom P	4% SE-30+ 6% QF-1, 80-100 Anachrom ABS	2% QF-1+ 3% OV-1, 80-100 Chrom W	5% QF-1, 80-100 Gas Chrom Q	3% OV-1 on 100- 120 Chrom W	1.5% OV-17+ 1.95% QF-1, 100-120 Chrom W
Methyl	0.51	0.62	0.64	0.47	0.56	0.47	0.45
Ethyl	0.66	0.66	0.72	0.55	0.64	0.57	0.47
Propyl	1.00	0.73	0.95	0.73	0.80	0.78	0.67
Isobutyl	1.24	0.77	1.11	0.86	0.92	0.95	0.75
Butyl	1.41	0.91	1.25	0.99	0.97	1.10	1.04
Isoamyl	1.81	1.03	1.50	1.20	1.20	1.31	1.13
Amyl	2.20	1.11	1.72	1.39	1.31	1.55	1.41

^aGLC conditions: 6' x 1/4" glass U-tube columns, 80 ml/minute nitrogen, and 180°C column temperature.

Determination of p-Values:

Individual volumes of acetonitrile, methanol, and dimethylformamide are equilibrated 1:1 with hexane at room temperature for 24 hours. A 0.1 ml aliquot of the hexane solution containing PCP-ether is pipetted into a 1 ml test tube and 0.1 ml of an equilibrated solvent is added. The two phases are thoroughly equilibrated by mixing 1 minute on a vortex mixer. After phase separation the upper hexane layer is ready for GLC. The p-value is the concentration of PCP ether in the hexane phase after partition divided by the concentration in the hexane before partition (see Table II).

TABLE II

p-Values of PCP-Ethers Determined by Single Distributions
Between Immiscible Phases at 25° C

PCP Ether	Acetonitrile: Hexane	Methanol: Hexane	Dimethylformamide: Hexane
Methyl ^a	0.62	0.61	0.44
Ethyl ^a	0.71	0.63	0.52
Propyl ^a	0.73	0.69	0.57
Isobutyl ^b	0.81	0.69	0.68
Butyl ^b	0.80	0.68	0.65
Isoamyl ^b	0.82	0.71	0.68
Amyl ^b	0.80	0.63	0.65

^ap-value consistently reproduced within 0.03.

^bp-value consistently reproduced within 0.02.

Results

Urine samples from individuals representative of the general population and from occupationally exposed individuals were analyzed in replicate, using this procedure. Replicates varied less than $\pm 5\%$ from their means over the range of 2.2 to 265 ppb PCP (Table III). The standard deviation decreased as the concentration of PCP increased.

TABLE III
Concentration Range of Pentachlorophenol in Human Urine

Sample	Origin	Replicate Range ^a , ppb	Mean ^b , ppb
1	General Population	2.2 - 2.3	2.2
2	General Population	2.7 - 2.9	2.8
3	General Population	2.9 - 3.0	2.9
4	General Population	5.0 - 5.3	5.1
5	General Population	5.2 - 5.5	5.3
6	General Population	10.6 - 11.2	10.8
7	Carpenter	22.2 - 25.5	24.1
8	Boat Builder	55.0 - 60.1	57.3
9	Sprayman	131 - 136	133
10	Sprayman	259 - 270	265

^aFour replicates of each sample.

^bVariation of the method was $\pm 5\%$.

Discussion

A number of precautions must be observed in using this procedure. All reagents, including the water used to prepare the NaOH, HCl, and MeOH solutions, must be extracted with hexane before use as they may be contaminated with PCP or other materials which interfere with the analysis. Deionized water is superior to distilled water with respect to the absence of PCP. Glassware should be washed with a dilute NaOH solution followed by deionized water and acetone rinses. Care should be taken not to allow contact between wooden or paper materials and glassware because peg boards and several brands of absorbent paper products have been found to contain PCP.

Preliminary extraction of the alkaline urine samples removes many interfering compounds that would subsequently be extracted from the acidic urine solution, including the neutral chlorinated hydrocarbons normally present in human urine (8). The volume of urine sampled and the amounts of acid and base

added have been selected to allow for urine with different pH values while excluding the largest amount of interfering material. It was found, as reported by Bevenue (9), that a more severe treatment of the urine before extraction, i.e., refluxing with concentrated acid, does not result in increased extraction of PCP. Also, additional extractions or extraction with a larger volume is unnecessary, because a single hexane extraction of acidified urine yields 90% of the PCP present. Five ml of urine is a volume convenient to manipulate; however, larger or smaller volumes of urine can be analyzed if the relative proportion of reagents to sample is maintained. If the suggested volumes are used, calculation of results is simplified because 1/2 the number of picograms of PCP derivative per μ l injected is equal to ppb of PCP present in the urine.

Hexane was chosen as the solvent for the extraction and alkylation reaction because it extracts the fewest interfering substances from urine while acting as an efficient extractant for PCP, because it can be purchased commercially in an ultrapure form, and because it produces very little response with an electron capture detector.

Caution must be used when handling the skin irritant diazoalkane reagents since both the reagents and the diazoalkane gases are extremely toxic and potentially explosive (10). Diazoalkane generation should be carried out in a hood; use of disposable gloves is desirable and manufacturers' recommendations for storage and handling should be closely adhered to. Diazoalkane solutions should not be pipetted by mouth. It is suggested that diazoalkane solutions be prepared fresh and only in the quantities needed; materials that give interfering GLC peaks are formed during storage. The original hexane-diazoalkane generating solution should not be stored in ground glass-stoppered containers; however, no hazard is involved in the 5 ml test tubes containing the PCP-hexane extract plus diazoalkane. Extended exposure to air destroys the diazoalkane reagents.

The efficiency of the alkylating reaction was constant over wide concentration ranges of reactant and PCP, five-fold for the diazoalkanes and one thousand-fold for PCP. The suggested concentration of alkylating reagents and the 20 min reaction time were selected to provide the analyst with a wide safety factor while insuring consistency of results.

Methanol containing 20% water effectively removes excess diazoalkane as well as many of the interferences present due to the diazotizing reagent. No loss of PCP ether results from this treatment and the volume does not have to be adjusted, as may be necessary when diazoalkane is removed by air or nitrogen jet.

The identity of a particular derivative of PCP found by GLC is confirmed with p -values. Availability and use of several derivatives increases the confidence that a correct identification has been made. p -Values (11, 12) are especially useful for confirmation of pesticides at levels which can only be determined by electron capture gas chromatography.

The analyst can avoid changing the conditions of GLC instrumentation, which may be set up for other analyses, by selecting a derivative with a retention value compatible with existing conditions. This flexibility allows the analyst to choose a derivative which yields good chromatographic data while avoiding the time-consuming process of instrument adjustment.

Summary

The procedure described is a rapid, reproducible, and flexible method for the determination of PCP in urine, which is also applicable to the analysis of water samples. The ability to confirm the identity of PCP and to quantitate accurately in the presence of interfering compounds is facilitated by the proper selection of one or more possible derivatives.

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